ANGÉLICA DE SOUZA GOUVEIA

DEFESA BIOQUÍMICA DE PLANTAS: ALTERAÇÕES DO METABOLISMO PELO FUNGO *Pochonia chlamydosporia* E NEMATOIDES

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Bioquímica Aplicada, para obtenção do título de *Doctor Scientiae*.

Orientador: Humberto Josué de Oliveira Ramos

Coorientadores: Leandro Grassi de Freitas
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RESUMO

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, junho de 2022. **Defesa Bioquímica de Plantas: Alterações do Metabolismo pelo Fungo *Pochonia chlamydosporia* e Nematoides**. Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes e Maria Goreti de Almeida Oliveira.

Os nematoides parasitas de plantas, como *Meloidogyne* e *Pratylenchus*, causam grandes perdas na agricultura. *Pratylenhcus brachyurus* ao infectar a soja desecandeia ativação de vias bioquímicas que podem contribuir para o sistema de defesa da planta. O estudo da cultivar BRS 7380 e MG/BR46 permitiu identificar a ativação da biossíntese de flavonoides, assim como, a síntese de fitoalexinas que contribuem para conter o desenvolvimento do patógeno. Além disso, o estudo da interação entre o nematoide e a raiz identificou que o patógeno desencadeia modificações na cascata hormonal, principalmente nos níveis de auxina que pode facilitar sua movimentação dentro da raiz ao longo do seu ciclo de vida. Para o controle de *P. brachyurus* o uso de microrganismos benéficos podem contribuir para a redução da população. A inoculação do fungo *Pochonia chlamydosporia* na cultivar MG/BR46 reduziu de forma eficaz a população final de *P. brachyurus*. No entanto, a aplicação desse fungo na cultivar BRS 7380 contribuiu para o aumento da população de nematoides. Nessa cultivar foi identificado maiores níveis de compostos fenólicos, lignina e síntese de fitoalexinas no período inicial da avaliação (7 e 21 dias) que pode ter prejudicado a colonização do fungo e um desbalanco nas respostas de defesa resultou no aumento da multiplicação do nematoide. O fungo *P. chlamydosporia* também foi eficiente na redução do número de ovos de *Meloidogyne javanica* em tomateiro. Análises bioquímicas da raiz permitiram identificar mecanismos indiretos utilizados pelo fungo que contribuiram para o controle do nematoide, como maior concentração de acido clorogênico e maior expressão dos genes FAL (Fenilalanina amônia-liase), LECHS2 (Chalcona sintase), PI1 (inibidor de protease) e RBOH. Além disso, com a análise metabolômica não-alvo foi identificado que a colonização do fungo na raiz proporcionou a modificação da abundância dos metabólitos que compõe a galha. A identificação de alguns desses metabólitos permitiu inferir que o aumento desses compostos pode prejudicar o desenvolvimento do nematoide. Na ausência de *M. javanica* esse fungo também alterou a abundância dos metabólitos que compõe a raiz de tomateiro, principalemente na avaliação de 24 dias e a redução de alguns compostos é importante para ocorrer a colonização fúngica. Nessa interação estabelecida detectou-se um efeito sistêmico, pois a abundância dos metabólitos das folhas foram alterados nas raízes colonizadas pelo fungo. As avaliações das diferentes interações entre planta, patógeno e microrganismo benéfico demonstram a complexidade dessas interações e a compreesão dessas pode auxiliar na elaboração de estratégias mais eficientes de controle.

Palavras-chave: *Pochonia chlamydosporia. Meloidogyne javanica. Pratylenhcus brachyurus.* Metabólitos. Raiz. Defesa.

ABSTRACT

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, June, 2022. **Biochemical Defense of Plants: Metabolism Alterations by the Fungus *Pochonia chlamydosporia* and Nematodes.** Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes and Maria Goreti de Almeida Oliveira.

Plant parasitic nematodes such as *Meloidogyne* and *Pratylenchu*s cause significant losses in agriculture. *Pratylenhcus brachyurus* when infecting soybean triggers the activation of biochemical pathways that may contribute to the plant's defense system. The study of the cultivar BRS 7380 and MG/BR46 allowed identifying the activation of flavonoid biosynthesis, as well as the synthesis of phytoalexins that contribute to containing the development of the pathogen. In addition, in the study of the interaction between the nematode and the root, it was identified that the pathogen triggers changes in the hormonal cascade, mainly in the levels of auxin, which can facilitate its movement within the root throughout its life cycle. For the control of *P. brachyurus*, the use of beneficial microorganisms can contribute to population reduction. Inoculation of the fungus *Pochonia chlamydosporia* in the cultivar MG/BR46 effectively reduced the final population of *P. brachyurus*. However, the application of this fungus on the cultivar BRS 7380 contributed to the increase in the population of nematodes. In this cultivar, higher levels of phenolic compounds, lignin, and phytoalexin synthesis were identified in the initial period of evaluation (7 and 21 days) which may have impaired the colonization of the fungus and an imbalance in the defense responses resulted in increased nematode multiplication. The fungus *P. chlamydosporia* was also efficient in reducing the number of *Meloidogyne javanica* eggs in tomato. Biochemical analyzes of the root allowed the identification of indirect mechanisms used by the fungus that contributed to the control of the nematode, such as higher concentration of chlorogenic acid and higher expression of PAL (Phenylalanine ammonia-lyase), LECHS2 (Chalcona synthase), PI1 (protease inhibitor) genes. and RBOH. In addition, with the non-target metabolomic analysis, it was identified that the colonization of the fungus in the root modified the abundance of the metabolites that constitute the gall. The identification of some of these metabolites allowed us to infer that the increase in these compounds can harm the development of the nematode. In the absence of *M. javanica*, this fungus also altered the abundance of metabolites that constitute tomato root, mainly in the 24 days evaluation, and the reduction of some compounds is important for fungal colonization to occur. In this established interaction, a systemic effect was detected, as the abundance of leaf metabolites was altered in the roots colonized by the fungus. The evaluations of the different interactions between plants, pathogens, and beneficial microorganisms demonstrate the complexity of these interactions and the understanding of these can help in the elaboration of more efficient strategies of control.

Keywords: *Pochonia chlamydosporia. Meloidogyne javanica. Pratylenhcus brachyurus.* Metabolomic. Root. Defense.

SUMÁRIO

O endófito de raiz nematófago *Pochonia chlamydosporia* altera o metaboloma do tomateiro

Inoculação de *Pochonia chlamydosporia* desencadeia respostas de defesas em raízes de tomateiro que reduzem a população de *Meloidogyne javanica*

Genótipo de soja infectado pelo nematoide das lesões radiculares, *Pratylenchus brachyurus*, desencadeia uma cascata de defesa vegetal dependente de ácido salicílico e aumenta a produção de flavonoides e fitoalexinas

Inoculação de *Pochonia chlamydosporia* promove redução diferencial na população do nematoide *Pratylenchus brachyurus* e alteração nos perfis de metabólitos dos genótipos de soja

INTRODUÇÃO GERAL

Microrganismos como fungos, bactérias e vírus habitam plantas e podem ser classificados como patogênicos e não-patogênicos. A simbiose desse segundo grupo pode ser dividida em relações mutualistas, comensais e neutras. Entre os organismos que estabelecem interações mutualísticas com plantas, os endofíticos colonizam raízes, caules ou folhas e proporcionam benefícios para o vegetal, como maior aquisição de nutrientes, tolerância a estresse abiótico (hídrico, salinilidade, presença de metal pesado) e indução de resistência contra fitopatógenos (Fadiji & Babalola, 2020).

Os endófiticos compreendem uma diversidade de fungos com alto potencial de biocontrole e a investigação desses tem proporcionado aumento do número de produtos biológicos no mercado. O fungo *Pochonia chamydosporia* é ingrediente ativo do produto comercial Rizotec, recomendado para o controle de nematoides parasitas de plantas. Esse fungo atua como saprófita, endofítico e é eficiente na promoção de crescimento vegetal de diferentes espécies (Monfort et al., 2005; Maciá-Vicente et al., 2009; Rosso et al., 2014).

Um dos mecanismos que auxilia nessa promoção de crescimento é o aumento radicular na presença do fungo que permite maior exploração do solo, com isso maior absorção e disponibilização de nutrientes para as plantas (Monteiro et al., 2018). Outros fatores como a produção de auxina pode contribuir para o incremento do desenvolvimento do vegetal, já que foi verificado que no cultivo de trigo com *P. chlamydosporia* ocorreu up-regulação de vários genes relacionados a esse hormônio (Larriba et al., 2015). *In vitro* esse fungo é capaz de produzir auxina. No entanto, a contribuição desse hormônio na interação planta-fungo ainda não foi completamente caracterizada (Zavala-Gonzalez et al., 2015).

P. chlamydosporia é capaz de parasitar ovos de diferentes gêneros de fitonematoides, dessa forma é amplamente utilizado como controle biológico (Manzanilla-López et al., 2017). O mecanismo direto de controle descrito para esse fungo utiliza a produção de enzimas (proteases, quitinases e lipases) com ação na casca dos ovos que permite o acesso do microrganismo ao interior desses (Esteves et al., 2009). O uso de microrganismos eficientes no controle de nematoides parasita de plantas e compreender a interação que ocorre nas raízes tem contribuído para o controle desse patógeno que ocasiona muitos prejuízos a agricultura, principalmente o gênero *Meloidogyne* e *Pratylenchus*.

Uma maio compreensão da interação de *P. chlamydosporia* com plantas para o controle biológico de nematoides parasitas de planta pode ser obtida com análises metabolômicas. Com essa ferramenta é possível verificar a composição de metabólitos na raiz nas diferentes fases de desenvolvimento do nematoide e a partir da identificação e quantificação desses pode ser possível estudos de vias relacionadas a reprodução do nematoide.

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CAPÍTULO 1

RESUMO

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, junho de 2022. ***O endófito de raiz nematófago *Pochonia chlamydosporia* altera o metaboloma do tomateiro.** Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes e Maria Goreti de Almeida Oliveira.

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O fungo endofítico *Pochonia chlamydosporia* é utilizado em produtos comerciais para o controle dos nematoides das galhas. Sua relação simbiótica com a planta também promove o crescimento dessas. Nós investigamos as alterações metabólicas que ocorrem em raízes e folhas de tomateiro quando ocorre a inoculação de *P. chlamydosporia*. Os perfis metabólicos de raízes e folhas foram contrastados por cromatografia líquida acoplada à espectrometria de massas (LC/MS) após 24 e 44 dias de interação planta-fungo. A simbiose resultou em maior biomassa vegetal em relação ao controle. Perfis fito-hormonais indicaram que a regulação do ácido jasmônico pode ser importante para estabelecer a interação endofítica. As plantas inoculadas também apresentaram alterações nos níveis radiculares de alguns compostos fenólicos e flavonoides. Maiores alterações metabolômicas no tecidos radiculares ocorreram no estádio inicial (24 dias após a inoculação), enquanto no estádio posterior os perfis foram muito semelhantes aos das plantas controle. Por outro lado, a inoculação do fungo promoveu uma resposta sistêmica, devido a alteração do metaboloma foliar nos dois períodos de avaliação. Essas alterações metabolômicas reforçam a ideia de que *P. chlamydosporia* é benéfico ao tomateiro, além de seu efeito nematicida.

Palavras-chave: *Pochonia chlamydosporia*. Perfil Metabolômico. Fitohormônios. Flavonoides. α-Tomatina.

ABSTRACT

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, June, 2022. **The nematophagous root endophyte *Pochonia chlamydosporia* alters tomato metabolome.** Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes and Maria Goreti de Almeida Oliveira.

The endophytic fungus *Pochonia chlamydosporia* has been used in commercial products to control root-knot nematode infections. Its mutualistic symbiosis also promotes plant growth and development. We investigated the metabolic changes that occur in tomato roots and leaves when in symbiosis with *P. chlamydosporia*. The metabolic profiles of roots and leaves were contrasted by liquid chromatograph coupled to mass spectrometry (LC/MS) after 24 and 44 days of plant-fungus interaction. The symbiosis resulted in higher plant biomass than controls. Phytohormonal profiles indicated that the jasmonic acid appears important for establishing the endophytic interaction. Inoculated plants also showed changes in the root levels of some phenolic and flavonoid compounds. Metabolomic alterations of root tissues occurred in the early stages (24 days after inoculation) of the endophytic interaction, while at later stages profiles were very similar to the control plants. Otherwise, inoculation promoted a systemic response as the leaf metabolome was altered for some defense compounds. These metabolomic changes reinforce the idea that *P. chlamydosporia* is beneficial to tomato in addition to its nematicide effect.

Keywords: *Pochonia chlamydosporia.* Metabolomic Profile. Phytohormones. Flavonoids. α-Tomatin.

1. Introduction

Mutualistic endophytes establish asymptomatic interactions which may alter the plant metabolism directly and indirectly (Mattoo et al., 2021; Adeleke et al., 2021). Changes in the endophyte also occur, mainly in its secondary metabolism, resulting in the production of compounds that can contribute to plant development, increased tolerance to abiotic stress, and increased resistance to pathogens (Fadiji & Babalola, 2020; Mishra et al., 2022; Ludwig-Muller et al., 2015). Deciphering transcriptomic and metabolomic changes caused by endophytic interactions help develop molecular models to understand and test the molecular details. These details improve the utility of inoculants in disease management and pathogen identification based on molecular markers (Sundin et al., 2016; Crandall et al., 2020; Wong et al., 2020). In addition, this tool helps to understand the metabolism of endophytes when cultivated in plants or a culture medium since the growing medium directly influences the production of secondary metabolites and many of these are investigated due to their therapeutic potential (Kusari et al., 2012).

In the biological control of plant parasitic nematodes, several nematophagous fungi are known to establish endophytic relationships with plants. Among these microorganisms, the fungus *Pochonia chlamydosporia* stands out and, in addition to being root endophytic, it has high efficiency in the control of plant parasitic nematode and it can promote plant growth of several crops of agricultural importance (Larriba et al., 2015; Escudero & Lopez-Llorca, 2012).

In roots from tomato (*Solanum lycopersicum* L.), *P. chamydosporia* colonizes the rhizoplane and establishes itself endophytically (Bordallo et al., 2002; Escudero & Lopez-Llorca, 2012; Dallemole-Giaretta et al., 2015). In this association, there is a change in the expression of root genes, which was verified 7, 14, and 21 days after the fungus inoculation. There was a specific modulation of stress-responsive transcripts related to the activation of defense pathways, probably necessary for the fungus to establish a style endophytic (Pentimone et al., 2019). Furthermore, the expression of defense genes in tomato leaves was altered after soil colonization with *P. chamydosporia* and plant exposure to biotic stresses (*Phytophthora infestans*, *Fusarium oxysporum f. sp. cubense*, *Meloidogyne incognita*, and *Pratylenchus goodeyi*), suggesting the occurrence of upward biochemical signals due to the presence of the fungus (Tolba et al., 2021). In addition, *P. chamydosporia* also reduces the flowering time of tomato, increasing the number and weight of fruits per plant (Zavala-Gonzalez et al., 2015). This precocious flowering was also detected in *Arabidopsis thaliana* and it was associated with expression changes in genes essential to flowering time (Zavala-Gonzalez et al., 2016).

From RNA seq data, it was verified that *P. chlamydosporia* induced alterations on the transcriptome of the tomato roots during endophytism. This remodeling of transcription was selective in directing the activation of defense pathways, probably necessary to allow its root colonization (Pentimone et al., 2019). Furthermore, some studies have evaluated metabolic responses to fungal inoculation (Escudero et al., 2014; Zavala et al., 2016; Suarez et al., 2020), including a possible systemic effect on tomato defense against root-knot nematodes (Ghahremani et al. 2019). However, characterization of altered compounds and pathways during the establishment of the fungus in the plant is still scarce. For this reason, in this study, metabolomic approaches by LC/MS were applied to identify alterations in compounds of tomato after inoculation of the fungus *P. chlamydosporia*. Changes in the root metabolites, the systemic effect of this interaction on leaves, and phytohormonal changes in the roots were evaluated in two periods. Evaluations occurred at 24 days, when there is maximum fungal colonization at the root (Pentimone et al., 2019) and at 44 days to investigate if the symbiosis between fungus and plant promotes benefits and alter the plant metabolome for a longer period of time. Molecular understanding of the interaction and benefits to the plant under colonization of this nematophagous fungus can provide important information that enables the expansion of their applications in agriculture.

2. Materials and Methods

2.1 Fungal and plant growth conditions

The fungus *Pochonia chlamydosporia* (Pc-10) used in this study was obtained from the mycology collection of the Laboratory of Control of Plant-parasitic Nematodes of the Federal University of Viçosa, Brazil and it is deposited in the culture collection “Coleção Octávio Almeida Drummond” of the Universidade Federal de Viçosa (Accession No. COAD 1781). It was cultivated in a solid medium consisting of 150 g of rice moistened with 40 mL of water and autoclaved for 20 minutes at 120 ºC. After this process, five discs from the fungal colony grown on potato dextrose agar for 21 days were added. For mass production, the fungus was kept for 21 days at 27 ºC and an aqueous suspension composed of chlamydospores and conidia was obtained. The number of spores was quantified using a Neubauer chamber.

The treatments consisted of cultivating tomato (*Solanum lycopersicum* L.) in the absence or presence of the *P. chlamydosporia.* For the fungus treatment, 5000 chlamydospores/g of soil were added to a 300 mL pot containing sterilized soil composed of C horizon clay soil and washed sand in the proportion of 1:1 (V:V) (**Table S1**). After five days, one tomato seedling (21 days) of the cultivar Santa Clara was transplanted to each pot. The experiment was incubated at a constant temperature of 25 ºC. Evaluations were carried out 24 and 44 days after transplanting. During each evaluation time, roots and leaves were collected and immediately frozen in liquid nitrogen and the plant tissues were stored in a freezer at -80 ºC. The experimental design was randomized and three biological replicates were used, each replicate consisting of a pool of three plants (nine plants by treatment). The growth evaluations measured the plant biomass, including root and shoot together. Growth promotion by fungal inoculation was evaluated relative to the control plants at the same time after transplanting.

2.2 *Pochonia chlamydosporia* detection

2.2.1 Soil

For detection of the fungus as inoculum at long of the experiment, 1g of soil was collected from each pot to determine the colony-forming units (CFU) of the fungus in the soil. The soil was diluted in sterile water, and the dilutions were plated on a semi-selective medium (Gaspard et al., 1990), with five repetitions per treatment.

2.2.2 Root

Root colonization by *P. chlamydosporia* at 21 and 44 days was detected by conventional PCR. The roots were surface sterilized with 1% sodium hypochlorite for 1 min and washed three times in sterile distilled water. Then, roots were ground in liquid nitrogen and for DNA extraction the CTAB (cetyltrimethylammonium bromide) method was utilized (Murray & Thompson, 1980). Conventional PCR reactions were performed for the VCP1 gene (VCP1-1F CGCTGGCTCTCTCACTAAGG;VCP1-2R TGCCAGTGTCAAGGACGTAG) using the PCR parameters (95°C for 5 min, 40 cycles at 95°C for 30 s, 60°C for 30 s and 72°C for 60 s) according to Lopez-Llorca et al. (2010). Reactions were performed in 20 µl with 100 ng from root DNA, 10 µM primers, and the PCR Master Mix Kit (Promega). An aliquot was used for amplicon detection by electrophoresis of 1% agarose.

2.3 Quantification of phytohormones by UHPLC-MS QqQ

The metabolites extraction was carried out by using 100 mg of fresh plant material (roots) macerated with nitrogen, and 400 µL of methanol, isopropanol, and acetic acid solution (20:79:1 v/v/v) were added. The extraction method was performed according to Vital (2019). The metabolites were separated by ultra-performance chromatography of the UHPLC type (Agilent), using a C18 column (50 mm x 1.0 mm ID, 1.7 µm particle, and 300 A), coupled online to the mass spectrometer triple quadrupole QqQ (Agilent). The equipment was operated in MRM (multiple reaction monitoring) mode. The mass of the precursor ion/fragment established was monitored by using standards for each molecule: ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), indolyl-3-acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA) (**Table S2**). IAA and ACC were scanned in the positive mode, while ABA, SA, and JA were in the negative mode. The data was analyzed using the Skyline software, and after obtaining peak area values, absolute quantification (ng/g of tissue) was performed using the standard curve of each phytohormone (Vital et al., 2019).

2.4 Evaluation of flavonoids and phenolic compounds by LC/MS

For the extraction of the compounds, 200 mg of the root was used, followed by the addition of 400 µL of extraction solution (methanol, isopropanol, and acetic acid solution (20: 79: 1), and the extraction method was performed according to Vital (2018). The metabolites were separated by ultra-performance chromatography of the UHPLC type (Agilent), using a C18 column (50 mm x 1.0 mm ID, 1.7 µm particle, and 300 A), coupled online to a triple quadrupole (QqQ) mass spectrometer (Agilent). The equipment was operated in MRM mode monitoring flavonoids and phenolics (**Table S3**), totaling 42 compounds. In this method, the retention times and MRM transitions (multiple reaction monitoring) generated for each standard were tabulated to create the transition list that was used as input to the Skyline package, allowing for quantitative analysis of specific compounds in the tomato root (Vital et al., 2018; Gómez et al., 2018).

2.5 Untargeted Metabolic profile by LC/MS

For the extraction of metabolites, 100 mg of plant material (roots or leaves) were macerated with liquid nitrogen and for each sample, 500 µL of extracting solution (methanol, isopropanol, and acetic acid) (20:79:1 v/v/v) was added. The samples were shaken for 20 seconds (4X) and kept in the sonicator for 10 min. Then, they remained for 30 min on ice, sonicated again for 10 min, and centrifuged at 6,000 x g for 10 min. These extraction procedures were repeated with the obtained pellet (Vital et al., 2019). The supernatant was analyzed by LC/MS using the nanoACQUITY UPLC system (Waters, Milford, MA, USA) with trap column and ProteCol GHQ303 C18 3.0 µm - 300 µm × 150 mm capillary column which operates with a flow rate of 5.0 µL.min-1, coupled to the Q-Tof mass spectrometer (micrO-Tof Q II, Bruker Daltonics Bremen, Germany)

During 85 minutes, ions were scanned for MS1 spectra in positive mode for masses between 100 and 1000 m/z, and between 50 and 1000 m/z for MS2 spectra. The Hystar software, version 3.2 (Bruker Daltonics, Bremen, Germany) was used for data acquisition, and the spectra were processed using the DataAnalysis program, version 4.0 (Bruker Daltonics, Bremen, Germany), with the default settings for metabolomics. The mass spectrometer was operated in Auto-MS2 mode, which obtained the MS2 spectra for the most intense ions found during the scan. Fragmentation lists were generated in the generic mgf format by the Data Analysis program (Gouveia et al., 2019).

Spectra were converted to mzXML format using the Proteowizard tool and they were utilized to compare the LC/MS profile of extracts by the XCMS platform (https://xcmsonline.scripps.edu) according to Gouveia et al. (2019). Alignments were performed following standard parameters for the HPLC/Q Tof system using 20 ppm for accuracy of the MS1 spectrum and the database of metabolites was *A. thaliana*. The paired comparison method was used to contrast the Control x *P. chlamydosporia* treatments. The table of metabolites containing the intensity of the detected ions was exported from the XCMS platform and statistical analysis was conducted in MetaboAnalyst (http://www.metaboanalyst.ca/) to which the data were submitted to logarithmic transformation. Significant differences between treatments were verified by Volcano Plot and the cut-off value was p-value < 0.05. Putative identification of the ions was done by comparing the fragmentation and the value of the m/z precursor with the NIST database.

2.6 Statistical analysis

The means and standard deviations of the results were calculated. Statistical analysis was performed for each evaluation period and the Student's t-test was used to distinguish between the two means by the R program version 3.6.0.

3. Results

3.1 Growth of tomato in response to the presence of *Pochonia chlamydosporia*

Pochonia chlamydosporia has been related as an endophytic fungus with the potential to promote plant growth mainly by capabilities to synthesize and secrete indoleacetic acid (IAA) in large amounts in a liquid medium (Monteiro et al., 2020). Thus, it was verified that root inoculation could increase the growth of tomato (*Solanum lycopersicum* L.). In both periods of interaction evaluated, *P. chlamydosporia* was able to promote plant growth. After 24 days of plant-fungus interaction, there was a 2.14-fold increase in plant biomass concerning the plant not inoculated with the fungus. For the second period, the fungus contributed to the increase in biomass 1.25 fold (**Figure 1A**). Soil samples were plated on a semi-selective medium and the fungus presence in both evaluation periods was confirmed (**Figure 1B**). Furthermore, the endophytic colonization of *P. chlamydosporia* in the roots was confirmed by presence of a PCR product for the VCP1 gene, specific for *P. chlamydosporia* (Figure S1).

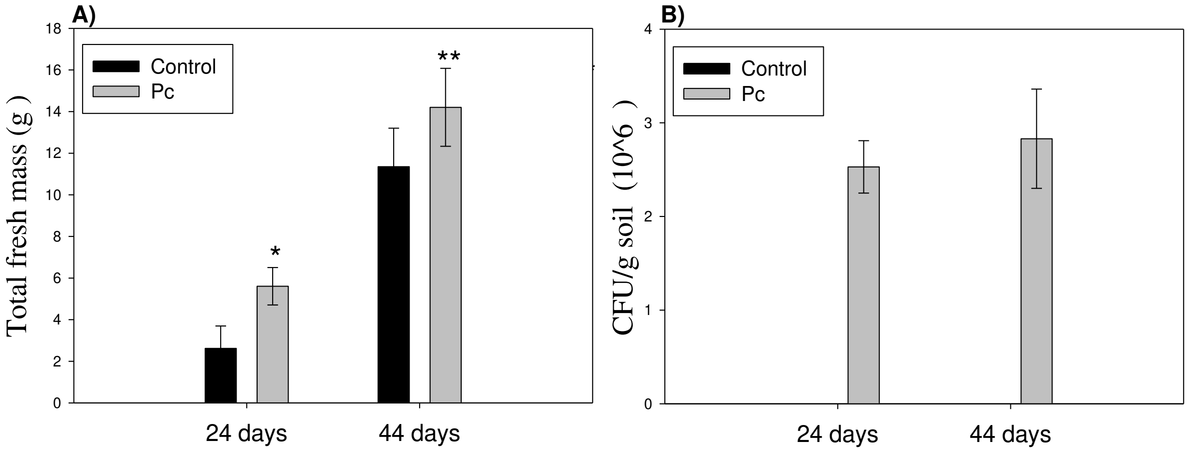

Figure 1: (A) Total fresh biomass of tomato in the absence or presence of *Pochonia chlamydosporia* (PC-10) 24 days and 44 days after application of the fungus. Mean followed by only one asterisk differs by the Student's t-test ($p < 0.05$) in the first evaluation period (24 days). Mean followed by double asterisk differs by the Student's t-test ($p < 0.05$) in the second evaluation period (44 days). Bars represent standard deviation (n= 9). **(B)** Colony-forming units (CFU) of the fungus *P. chlamydosporia*, in soil, applied at concentrations of 0 chlamydospores/g soil (Control) and 5,000 chlamydospores (Pc)/g soil.

3.2 Colonization of tomato roots by *Pochonia chlamydosporia* alters jasmonic acid levels

The mutualistic interaction between plants and microorganisms can be regulated by the action of phytohormones that establish communication between them. When analyzing these compounds in tomato roots in the presence of *P. chlamydosporia*, in the two evaluated periods, there was no statistical difference in 1-aminocyclopropane-1-carboxylic acid, salicylic acid, abscisic acid, and indole-3-acetic acid concentrations, when compared to the control treatment (**Figure S2**). On the other hand, the fungus presence induced significant changes in the concentration of jasmonic acid in the evaluated periods (**Figure 2**). It was possible to highlight a remarkable 47.23-fold reduction in JA levels at the first evaluation (24 days) and an increase of 3.75-fold in the second (44 days) (**Figure 2**). Thus, modulation of the JA levels may be necessary for allowing the fungal colonization of the tomato roots.

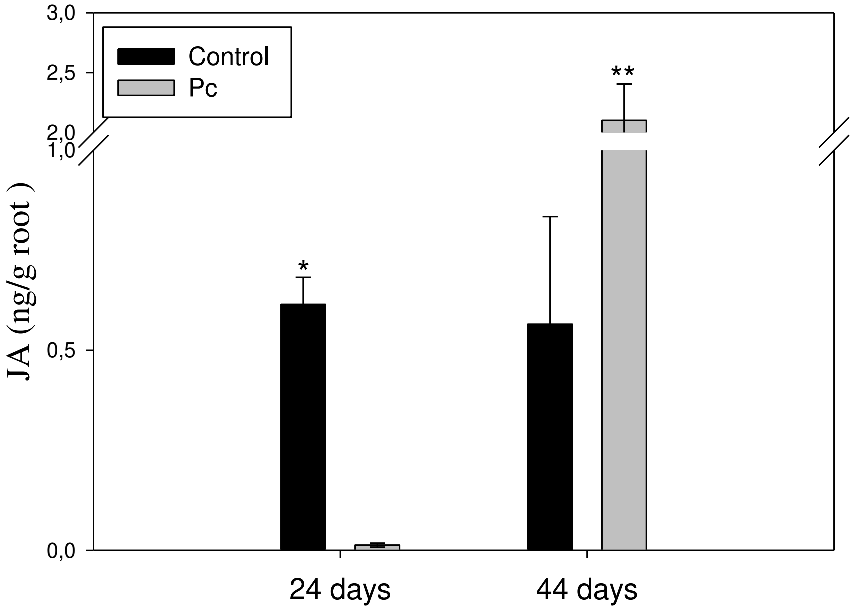

Figure 2: Quantification of jasmonic acid from tomato roots colonized by *Pochonia chlamydosporia*. Mean followed by only one asterisk differs by the Student's t-test ($p < 0.05$) in the first evaluation period (24 days). Mean followed by double asterisk differs by the Student's t-test ($p < 0.05$) in the second evaluation period (44 days). Pc: *Pochonia chlamydosporia*.

3.3 Evaluation of flavonoids and phenolic compounds by LC/MS

A typical response to fungal infection is the induction of defense compound production, including activation of the flavonoid pathway. Thus, the LC/MS method was applied to analyze some phenolic compounds and flavonoids. It was possible to identify some compounds whose relative abundance is altered due to the presence of the fungus in the root (**Figure 3**). After 24 days of interaction, the root colonized by the fungus had a more amount of rutin and chlorogenic acid (**Figure 3A and 3E**), while this difference was not significant at 44 days of interaction. However, the glycoconjugate isovitexin, a flavone glycoconjugate of apigenin (the apigenin-6-C-glucoside), remained more abundant in the root colonized by the fungus during the evaluation process (**Figure 3B**). For Hesperetin, a flavanone aglycone, the fungus provided opposite results in the evaluation periods since, in the first period, there was a reduction of this compound, and in the second, there was a greater abundance in the colonized roots (**Figure 3C**). While Hesperidin, a flavanone glycoside containing hesperetin (the aglycone form) and sucrose, was identified only in samples referring to the second evaluation period, and the presence of the fungus contributed to the increase of this compound in the root (**Figure 3D**). Regarding the coumaric acid, the fungus colonization did not result in significant changes (**Figure 3F**).

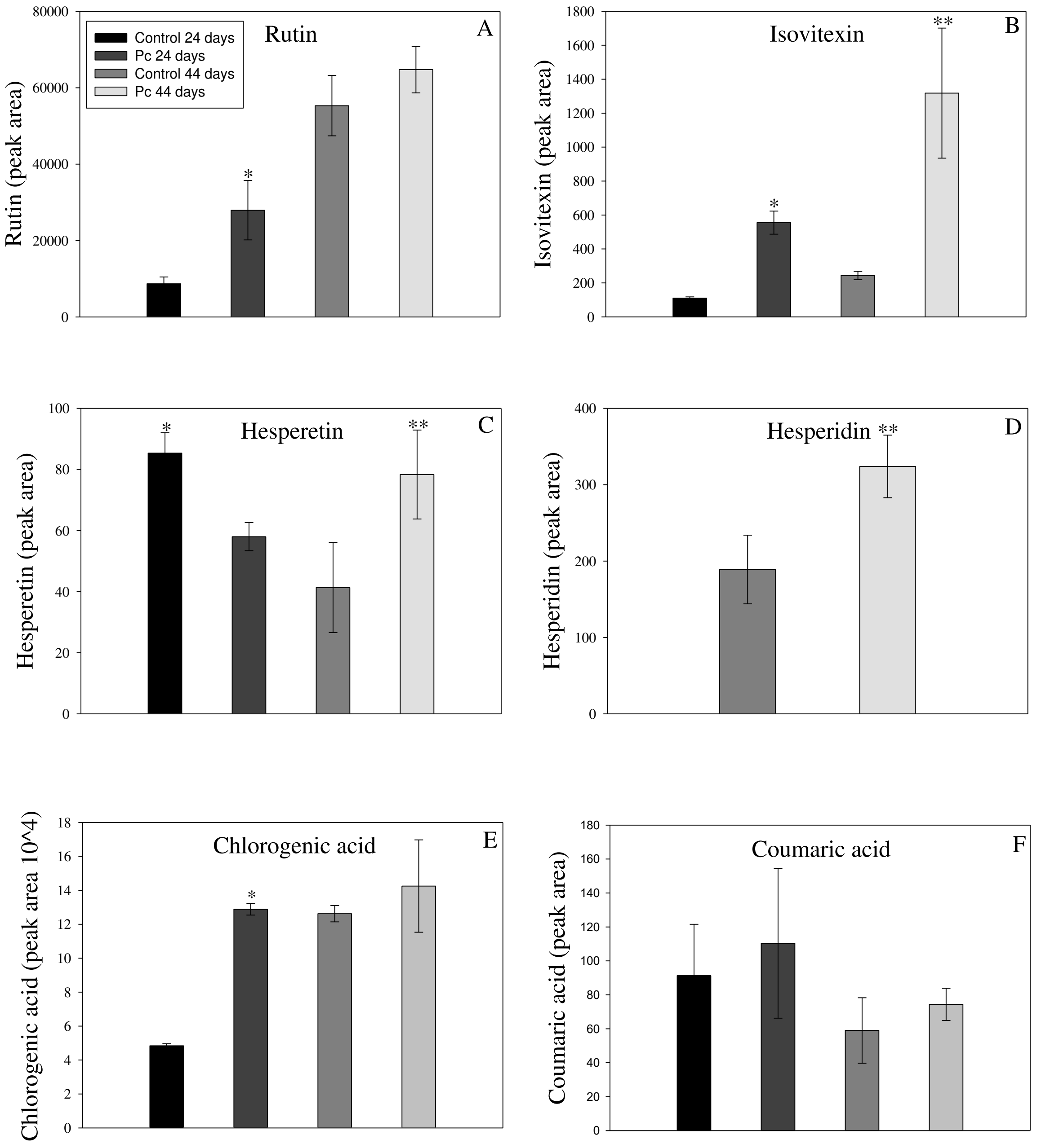

Figure 3: Relative abundance of flavonoids and phenolic compounds in tomato roots colonized or not by the fungus *Pochonia chlamydosporia*. Mean followed by only one asterisk differs by the Student's t-test ($p < 0.05$) in the first evaluation period (24 days). Mean followed by double asterisk differs by the Student's t-test ($p < 0.05$) in the second evaluation period (44 days). The

graphics refer to the following compounds: **A**: rutin; **B**: isovitexin; **C**: hesperetin; **D**: hesperidin; **E**: chlorogenic acid; **F**: coumaric acid.

3.4 Evaluation of metabolites in the roots

As the colonization of the tomato roots by *P. chlamydosporia* significantly altered the levels of jasmonic acid (**Figure 2**), flavonoids, and phenolic compounds (**Figure 3**), the untargeted metabolic profiles were evaluated by LC/MS to verify the magnitude of alteration in the metabolism due to the presence of the fungus.

Firstly, all detected metabolites from the LC/MS profiles were compared to verify changes in root metabolites during the fungus colonization process. Principal component analysis (PCA) and partial least squares discriminant analysis (PLS-DA) were utilized to evaluate the metabolomic data. The PCA plot from the first-period evaluation revealed a separation between plants inoculated and control, with 71% of the total variance observed, where the main components PC1 and PC2 explain 44.6% and 26.4% of the variance, respectively (**Figure 4A**). The separation between the groups indicates that the abundance of root metabolites colonized by the fungus can be distinguished from the control plant. The supervised method PLS-DA, which seeks to maximize the separation between groups using the most discriminant variable, also showed group separation ($Q^2 = 0.72007$ and $R^2 = 0.99993$), and components 1 and 2 explained 67.4% of the total variation between treatments (**Figure S3**). Thus, the fungal colonization altered the plant metabolism at the first stages of interaction.

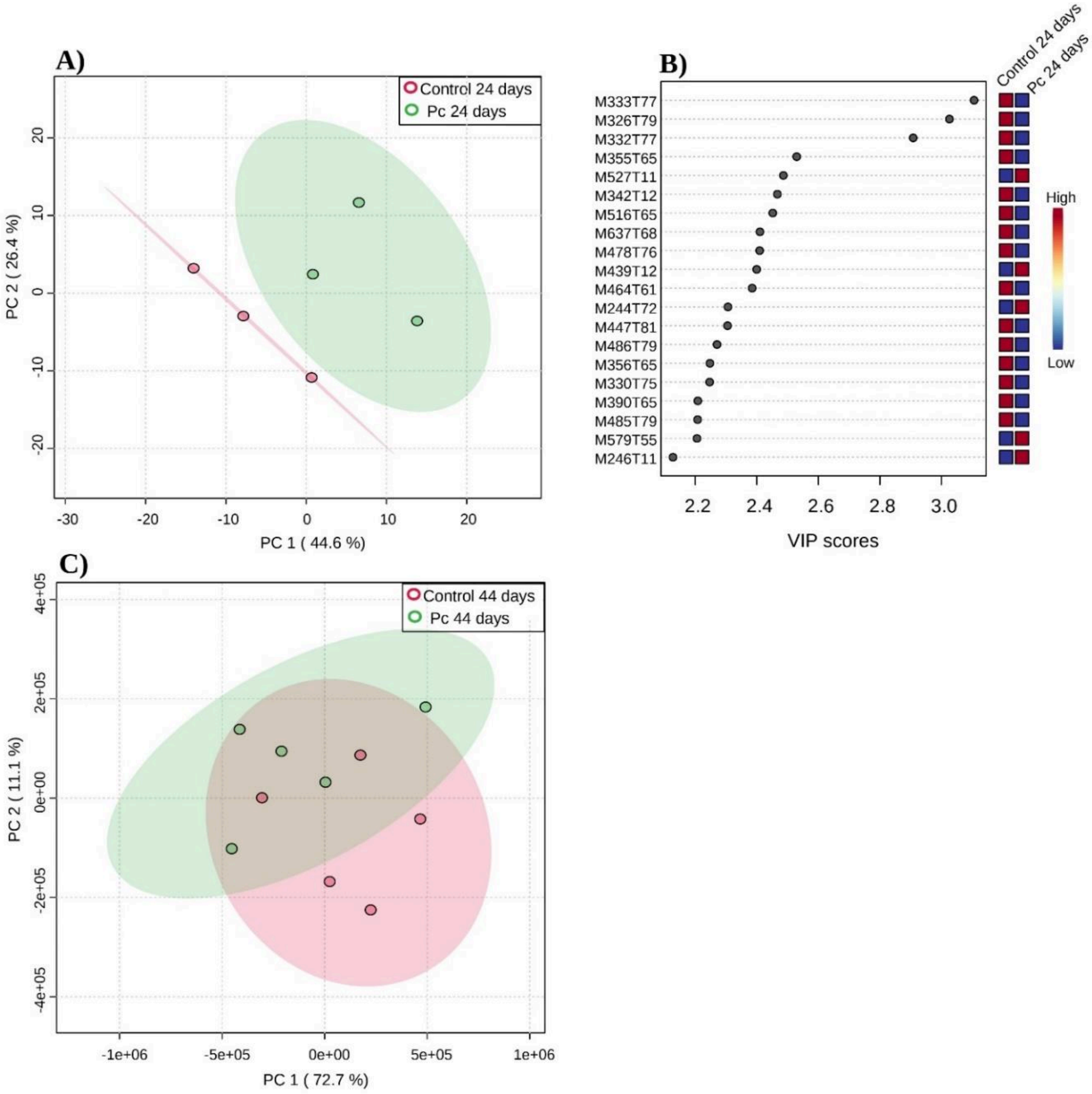

Figure 4: **A)** Principal component analysis (PCA) to comparison of the LC/MS metabolic profile of tomato root extracts after 24 days of interaction with *Pochonia chlamydosporia* with untreated plants (control). **B)** Variable importance in projection (VIP) score plot for the top 20 most important metabolite features identified by PLS-DA. The box indicates the relative concentration in colonized and uncolonized tomato roots by *P.chlamydosporia* at 24 days. C) PCA comparing the LC/MS metabolic profle of tomato root extracts after 44 days of interaction with *P. chlamydosporia* with untreated plants (control).

As these models revealed distinct groupings between the two treatments, the most altered ions contributing to the group separation were classified by the VIP score (Variable importance in projection) (**Figure 4B**). In the first period of the metabolic analysis, it is possible to identify 20 ions important separating groups in the PLS-DA. Among these ions, M464T61 and M447T81, are exclusive of roots referring to the control plant and the ion M579T55 was detected only in roots colonized by the fungus (**Figure 4B**).

When the metabolomic profiles from plants after 44 days under fungal colonization of the tomato roots were compared, it was possible to observe less variation in plant metabolism compared to the control, as demonstrated in the region of intersection formed in the PCA (**Figure 4C**). The components (PC1 and PC2) explain 83.8% of the data variance. The PLS-DA method, on the other hand, was not well validated. Therefore, it was not used to assess data referring to the second evaluation period. Thus, these results indicate that the plant metabolism was barely affected by fungal colonization in a later stage of interaction.

Accordingly, when the Volcano Plot method was applied to profiles from the first period (24 days), 216 ions were observed with a statistical difference ($p < 0.05$), and it was revealed which ones of them had increased or reduced abundance in response to colonization of roots by the fungus (**Figure S4A**). Otherwise, for the second period, the abundance of only 20 ions was statistically different ($p < 0.05$) and fungus colonization contributed to the reduction of most of these metabolites (**Figure S4B**). Therefore, it is confirmed that after 44 days of interaction, plant metabolism was little affected by fungal colonization compared to non-colonized plants.

Ions showing significant changes in abundance between treatments were checked for the presence of high quality MS2 spectra, which were compared with spectra present in metabolite libraries using the NIST package. Thus, it was possible to identify four compounds from the first period and one from the second evaluation period (**Table 1**). The identified metabolites belong to the steroid and carbohydrate class, highlighting compounds derivatives of the tomatidine, which showed a reduction in their abundance in response to fungal colonization.

Table 1: Putative identification of unregulated metabolites from tomato roots colonized by *Pochonia chlamydosporia*.

Identification	Fold change*	Theoretical (m/z)	Observed (m/z)	p-valor	Interaction (days)
Tomatidine	-2.34	416.3523	416.347	0.0375	24
Deydrotomatidine	-1.20	414.3338	414.3366	0.0472	24
Tomatidine-galactose	-3.806	578.4073	578.3803	0.0032	24
Sucrose	+0.22	365.1054	365.10065	0.035	24
Tomatidine	-3.00	416.3523	416.32892	0.0384	44

* Fold change with positive signal (+) indicates an increase and negative (-) indicates a decrease in the abundance.

3.5 Evaluation of metabolites in the leaves

Metabolic changes in leaves due to root colonization by *P. chlamydosporia* were investigated with 24 and 44 days of interaction to assess the systemic effect of fungus colonization. The PCA plot from leaves collected in the first evaluation period (24 days) revealed that the replicates of the two treatments were grouped separately with 66.3% of the total variance between the two groups, and PC1 and PC2 explained 48.9% and 17.4% of the variance, respectively (**Figure S5A**). The separation between the groups indicates that the abundance of leaf metabolites from plants colonized by *P. chlamydosporia* in the root can be distinguished from the control plant. The supervised method, PLS-DA, which maximizes the separation between groups, was well validated (Q^2 = 0.68661 and R^2 = 0.93904), and components 1 and 2 explained 62.6% of the total variation between treatments (**Figure 5A**). This model also revealed that the replicates of each treatment formed distinct groups, and the determinant metabolites in the separation were classified by the VIP score (Variable importance in projection) (**Figure 5B**).

Leaf metabolites within 44 days of fungus interaction with the root were also altered as well as in the first evaluation period. The PCA method revealed the separation of treatments with 67.2% of the total variance between the two groups, where PC1 and PC2 explained 45.2% and 22% of the variance, respectively (**Figure S5B**). The PLS-DA method was well validated

($Q^2 = 0.80554$ and $R^2 = 0.99998$), components 1 and 2 explained 60.4% of the total variation between treatments (**Figure 5C**), and the determinant metabolites in separation were classified by VIP score (Variable importance in projection) (**Figure 5D**). Therefore, global analysis of LC/MS profiles indicated that the leaf metabolism might have been affected, in both evaluation periods, in response to the root colonization by *P. chlamydosporia*.

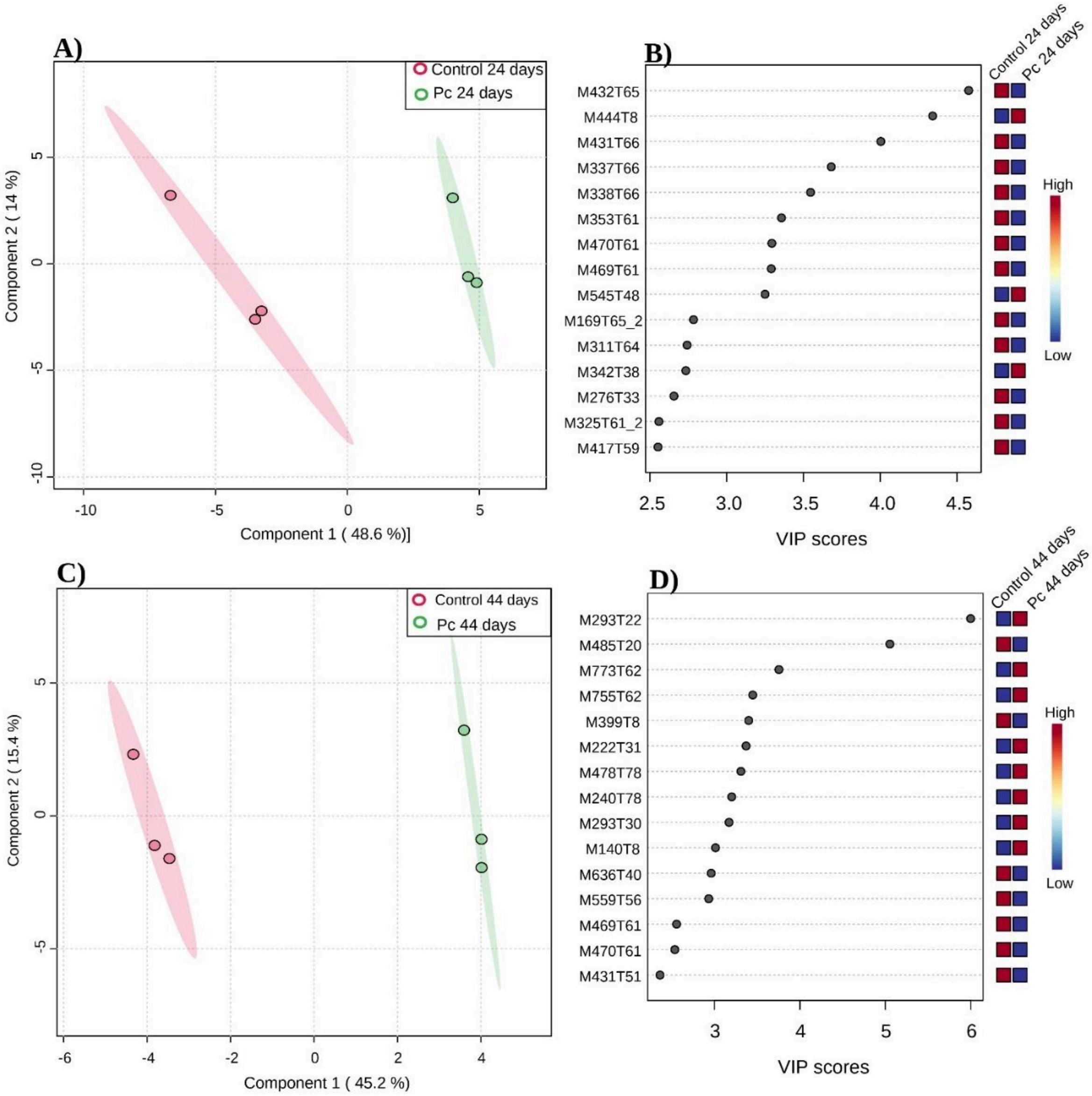

Figure 5: **(A)** Clustering analysis by partial least squares discriminant analysis (PLS-DA) in comparing the metabolic profile of LC/MS of a leaf extract from tomato not-colonized or colonized by the fungus *Pochonia chlamydosporia* after 24 days. In **(B)** most responsive ions from PLS-DA from LC/MS profiles from tomato not-colonized or colonized by the fungus *P. chlamydosporia.* **(C)** Clustering analysis by PLS-DA in comparing the metabolic profile of LC/MS of a leaf extract from tomato not-colonized or colonized by the fungus *P.*

chlamydosporia after 44 days. In **(D)** most responsive ions from PLS-DA from LC/MS profiles from tomato not-colonized or colonized by the fungus *P. chlamydosporia* after 44 days.

The Volcano Plot from the first period (24 days) showed 114 ions with significantly different abundance ($p < 0.05$) on the leaves when the roots were colonized by the fungus (**Figure S6A**). In the second period, the abundance of 184 ions showed a statistical difference ($p < 0.05$), and the colonization of the fungus caused an increase in specific metabolites and a reduction in the others (**Figure S6B**).

Fragmentation spectra and precursor values (m/z) of metabolites with statistical significance were compared with spectra present in metabolite libraries using the NIST package. From the library, it was possible to annotate some compounds from the two evaluated periods (**Table 2**).

Table 2: Putative identification of deregulated metabolites from leaves of plants that had their roots colonized by *Pochonia chlamydosporia.*

Identification	Fold change	Theoretical (m/z)	Observed (m/z)	**p-valor**	**Interaction (days)**
2-Monolinolein	-12.30	337.2737 ($[M+H-H_2O]^+$)	337.25629	0.0265	24 days
Tomatidine	+0.40	416.3523	416.33221	0.0404	24 days
Monolinolenin	+2.47	353.2686 $[M+H]^+$	353.24954	0.03776	44 days
Monolinolenin	+1.68	335.2581 $[M+H-H_2O]^+$	335.2463	0.00450	44 days

* Fold change with positive signal (+) indicates an increase and negative (-) indicates a decrease in the abundance.

4. Discussion

The fungus *Pochonia chlamydosporia* is used in agriculture to control plant parasitic nematodes, and it is capable of promoting plant growth. Insights about plant-fungus interaction have also emerged from transcriptome studies and metabolomic analysis of root exudates (Pentimone et al., 2019; Escudero et al., 2014). In addition to regulating plant growth and development, hormones provide responses to control the colonization of beneficial microorganisms (Yan et al., 2019). Thus, hormones were analyzed in two periods of colonization (24 and 44 days), the first one corresponds to the period of the maximum degree of root colonization by the fungus (Pentimone et al., 2019) and the second is one to evaluate later stages of interaction. We observed that modulation of the JA levels at long of tomato (*Solanum lycopersicum* L.) colonization might determine the fungal endophytic association. Zavala-Gonzalez et al. (2016) observed that colonization of *Arabidopsis thaliana* roots by *P. chlamydosporia* is modulated by jasmonate signaling.

At the first period evaluation, there was a reduction of jasmonic acid in the root colonized by the fungus. The reduction of this hormone was also found in *A. thaliana* root when colonized by this fungus for 21 days (Zavala-Gonzalez et al., 2016), and in tomato root transcriptome there was some down-regulation of genes related to the biosynthesis of jasmonic acid in the presence of the fungus (Pentimone et al., 2019). Based on these data, it is possible to suggest that, for the establishment of *P.chamydosporia*, it may be necessary to reduce the plant's defenses, such as the production of jasmonic acid.

In order to establish a favorable interaction, plants have mechanisms to avoid excessive colonization by beneficial microorganisms since the increase in nutrients transferred to the symbiont can limit its growth. Jasmonic acid is a critical component of these mechanisms, being able to activate regulatory cascades to limit the growth of microorganisms in the root (Hause & Schaarschmidt, 2009), which would justify the increase of this hormone in tomato root in the second period (44 days) in the presence of the fungus. The role of this hormone in regulating colonization was also demonstrated in plants deficient in jasmonic acid production, which were more colonized by *P. chlamydosporia* (Zavala-Gonzalez et al., 2016).

Flavonoids are secondary metabolites that play multiple roles in the plant's response to abiotic and biotic stress. They can be produced by plants and released into the rhizosphere, as a strategy to acquire nutrients from the soil and it stands out for their antioxidant activity that provides stress protection by acting as ROS scavengers (Cesco et al., 2012; WinkelShirley, 2002; Nakabayashi et al., 2014). The association of microorganisms with plants can result in changes in these compounds, for example, the presence of growth-promoting bacteria (Enterobacter 15S and Pseudomonas 16S), which influenced the metabolism of the rhizosphere microbiome, being flavonoids and phenolics the most frequently detected metabolites (Zuluaga et al., 2021).

The increase in root rutin in the first evaluation period due to the presence of the fungus may contribute to the protection of the plant against nematode attack since this compound *in vitro* causes the mortality of juveniles (J2) of *M. incognita* (Bano et al., 2020). Furthermore, tobacco rutin is a regulatory compound that promotes root growth and increases root hair length

(Park et al., 2019). Thus, the increase in rutin levels provided by *P. chlamydosporia* may be related to an increase in the root system observed in different studies with this fungus (Coutinho et al., 2021; Zavala-Gonzalez et al., 2016; Dallemole-Giaretta et al., 2015). Likewise, the compound apigenin-7-O-glucoside also causes mortality in juveniles of *M. incognita* (Bano et al., 2020). In addition, isovitexin (apigenin-6-O-glucoside) also showed high levels in both periods in response to the fungus. Due to the similarity of these compounds, it is possible that isovitexin also may affect root-knot nematodes.

Increased chlorogenic acid in roots with the fungus may be important for the control of plant-parasitic nematodes since plants resistant to root-knot nematodes have a greater amount of this phenolic compound in roots (Sato et al., 2019; El-Sappah et al., 2021). Besides that, changes in the flavonoid (hesperetin) profile due to the presence of *P. chlamydosporia* may also contribute to the control of fungal diseases. Małolepsza et al. (2017) have also observed that hesperetin, *in vitro* conditions, may inhibit the growth of *Rhizoctonia solani*. So, there is the possibility of roots with a higher concentration of this flavonoid reducing the growth of this pathogen. Interestingly, tomato treated with chitosan lead to the accumulation of phenolic compounds and hormones related to plant growth and defense (Suarez-Fernandez et al., 2020). We also observed changes in JA and some phenolics in response to fungal infection. Thus, the regulatory cascade affecting the phenylpropanoid pathway may be a direct or indirect effect of fungal colonization, leading to the production of compound signal compounds such as chitosan (Escudero et al., 2017; Suarez-Fernandez et al., 2020).

The untargeted metabolites profiles by LC/MS were also analyzed at two periods of colonization (24 and 44 days) to evaluate global alterations in the plant metabolism under *P. chlamydosporia* colonization. In the first period, presence of the fungus changed the root's metabolite profile, while in the second period, the profile of the colonized root is very similar to that of the non-colonized root. This significant change related to the first period may be due to the phase of the establishment of the microorganism, as it needs to develop maintaining an asymptomatic interaction, which may require further changes in metabolic pathways, as verified by jasmonic acid levels.

In the root colonized by the fungus, there was a reduction in the abundance of three intermediates (tomatidine, deydrotomatidine, and tomatidine-galactose) of the α-tomatin biosynthesis pathway during the first period of interaction. This glycoalkaloid is found in the root, stem, leaf, flower, and green fruit of tomato (Roddick, 1974). It can interact with OH groups from sterols present in the fungal plasma membrane, causing the formation of pores and resulting in the loss of cell integrity, revealing its antifungal potential (Steel et al., 1988). Therefore, reducing the biosynthesis of α-tomatin may be necessary for *P. chlamydosporia* colonization.

Differentially expressed genes were identified in the transcriptome of tomato root cultivated with *P. chlamydosporia* (DSM 26985), which supports the hypothesis that plants treated with the fungus can reduce α-tomatin production. Genes related to UDP-glycosyltransferases and transferase activity (hexosyl group transfer) were down-regulated in the presence of the fungus after 4, 7 and 21 days of colonization (Pentimone et al., 2019). The downregulation of genes related to essential enzymes in α-tomatin biosynthesis and the lower abundance of intermediates in this pathway (**Figure 6**) suggest a lower production of this glycoalkaloid, favoring fungal colonization. Mycorrhizal fungi, such as *Rhizophagus irregularis* colonize tomato roots efficiently when there is a low concentration of α-tomatin, causing down-regulation of the GAME1 gene (galactosyltransferase), an important enzyme for the synthesis of this glycoalkaloid (Casarrubias-Castillo et al., 2019).

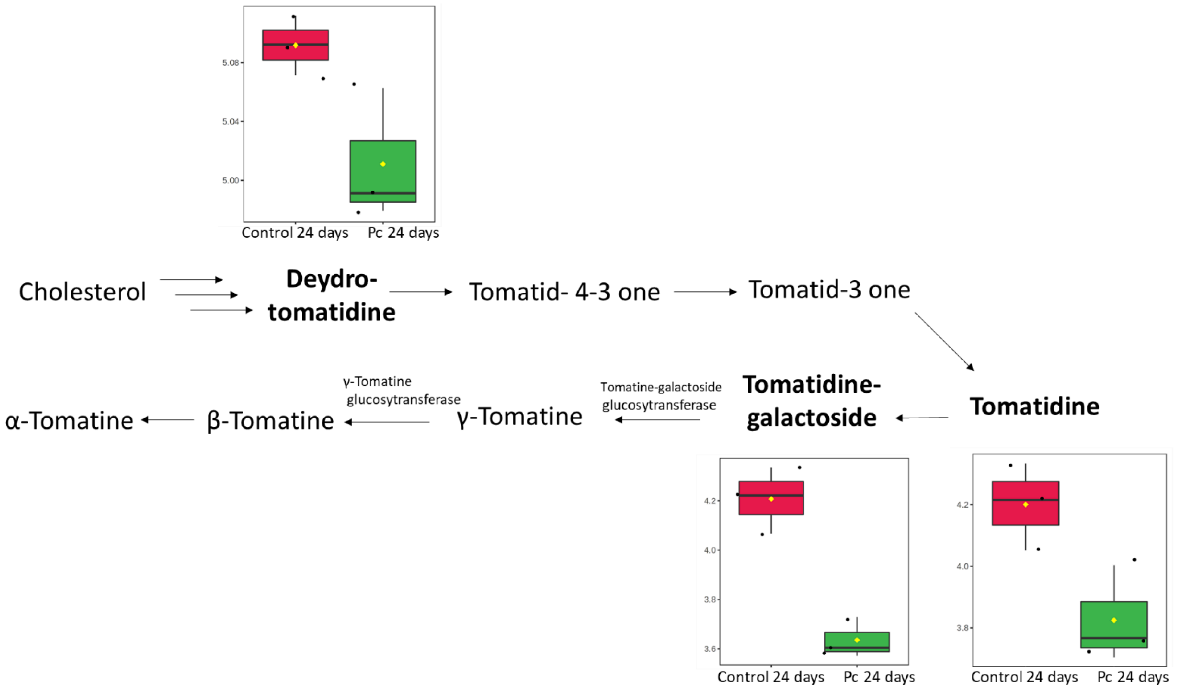

Figure 6: Biosynthesis of α-tomatin with emphasis on possible enzymes that presented their genes down-regulated in tomato roots cultivated with *Pochonia chlamydosporia* (DSM 26985) in the transcriptome study and in bold letters the intermediates identified in the LC/MS-MS profile that presented lower abundance in the root extracts colonized by the fungus. Red bar refers to control 24 days and green bar to treatment with *P. chlamydosporia* 24 days.

In addition to the possible reduction in α-tomatin biosynthesis, the fungus *P. chlamydosporia* has the potential to hydrolyze this compound. Tomato pathogenic fungi are, in general, less sensitive to α-tomatin than non-pathogenic fungi due to the production of specific enzymes, known as tomatinases. This enzyme hydrolysis of α-tomatin releases tomatidine and licotetraose, which do not have fungicidal potential (Ito et al., 2004). Thus, amino acid sequences related to the tomatinase enzyme were searched in the set of putative proteins of *P. chlamydosporia* (PC-170) to verify its presence in the genome. Sequence alignments using BLAST indicated that the *P. chlamydosporia* protein XP_018136485.1 had 32 % identity with a *Fusarium oxysporum* tomatinase, and analysis of functional domains in this sequence using InterProScan indicated the presence of a glycosylhydrolase domain from family 10 (GH10). It is described that proteins that belong to this family have tomatinase activity since α-tomatin has O-glycosidic bonds that this group of enzymes can hydrolyze. Thus, the metabolomic data and genomic information indicated that the establishment of endophytic in the fungus might be favored by its ability to secrete enzymes that hydrolyze α-tomatin.

In the first evaluation period, root extracts that contained the fungus had the 365T8 ion in greater abundance. According to its fragmentation pattern and the value of the m/z precursor, this ion was noted as sucrose. In mutualistic interactions between fungi and plants, plants provide carbon in the form of sugars to the fungus and the microorganism provides nutrients, mainly phosphorus (P), increased tolerance to abiotic stress and disease resistance to plants. Part of the carbon supplied to the fungus is destined for the production of chitin for the extension of hyphae in the soil, which allows greater exploration of the area and increased P uptake by the plants (Kiers et al., 2011; Garcia Garrido & Ocampo, 2002).

The increase in sucrose in the roots with *P. chlamydosporia* corroborated with the observations that, during the mutualistic relationship between the tomato and the fungus, there is a nutrient exchange promoting the growth and benefits for both partners (Monteiro et al., 2018; Manzanilla-López et al., 2011). Furthermore, the genome of Pc-10 (unpublished data) contains essential proteins for the uptake of sugars made available by the plant, 10 proteins referring to monosaccharide transporters, 2 sucrose transporter proteins and 15 proteins noted as sugar transporters. Based on this information, it is possible that plants and fungi actively exchange nutrients, resulting in better plant development (Rosso et al., 2014; Maciá-Vicente et al., 2009; Dallemole-Giaretta et al., 2015).

The ViP score analysis for the first period identified important ions for separating groups, and among these, three ions were exclusive to a given treatment. The ions M464T61 and M447T81 were exclusive of non-colonized roots, and the ion M579T55 was detected only in roots colonized with the fungus. Identifying these ions was not possible, but the regulation of these metabolites may be important for the fungus to establish itself in the plant since the first period (24 days) corresponds to the phase of maximum colonization (Pentimone et al., 2019). For this to occur without causing any visible disease symptoms, a balanced antagonism is necessary, in other words, a balance between the virulence of the endophytic fungus and the plant's defense responses. Thus, some plant compounds may be inhibited, and the fungus synthesizes others to aid in its colonization (Schulz & Boyle, 2005; Yan et al., 2019). In the second period (44 days), with well-established endophytism, there were no ions exclusive to a particular treatment.

Studies have verified that the fungus can cause adverse effects on the plant at the beginning of colonization. Root cells show some adverse reactions to colonization by the fungus (Escudero et al., 2014). However, they do not cause evident disease symptoms as they do for pathogenic fungi. That is why it has been written that colonization occurs asymptomatically. In fact, metabolic changes were more evident in the first stages of the plant-host interactions. In accordance, Escudero et al. (2014) observed that root exudates from plants under *M. javanica* infection contained more aromatic amino acid compounds than control and colonized by *P. chlamydosporia* plants.

In addition to the metabolic changes that the fungus causes in the root, there is also an alteration in the leaf metabolite profile. This change may also contribute to defense strategies used against biotic and abiotic stresses. Zavala-Gonzalez et al. (2016) demonstrate that *P. chlamydosporia* colonizes *A. thaliana,* including root cell invasion, affecting the shoots, reducing flowering time and stimulating plant growth.

However, this is the first study showing alterations in the metabolic profiles of the leaves in the plants whose roots were colonized by *P. chlamydosporia*. This profile remains different up to 44 days of interaction, which arouses interest in understanding the role of these changes in defense mechanisms. Among these altered metabolites in the leaves, in the first period, 2-monolinolein was identified, a monoacylglycerol derived from linoleic acid, a compound similar to monolinolenin (M335T64 and M353T61) was identified in the second period, which presents only one more instauration than 2-monolinolein. These compounds have already been detected in leaves of other plant species, but there are no reports on their role in plant metabolism (Conforti et al., 2010; Padmapriya & Maneemegalai, 2016).

Finally, the fungus interaction in the root also increased the abundance of tomatidine in the leaves, an intermediate compound for the synthesis of α-tomatin. This compound acts as a plant defense mechanism, and in tomato leaves, it was observed that the attack by *Spodoptera litura* increases the production of α-tomatin. Furthermore, the addition of this compound to the artificial diet reduced the growth and altered the morphology of *S. litura* (Li et al., 2019; Koh et al., 2012). The abundance increase of an intermediary of the α-tomatin biosynthesis pathway may reflect the activation of this pathway, so the addition of *P. chlamydosporia* to the soil may favor the plant's defense mechanisms in the shoot. Thus, alterations in the leaf metabolism in plants under fungal interactions may also contribute to plant defenses and improve growth.

5. Conclusion

The metabolomic study of the *Solanum lycopersicum* plants wich *P. chlamydosporia* provided important information on this plant-fungus interaction**.** On the roots, the most expressive metabolomic alterations occurred in the first period (24 days), which corresponds to the endophytic establishment phase. Since this establishment occurred in the second period (44 days), the root metabolome of treated and untreated plants with the fungus was very similar. Alterations in the metabolome, such as the increased flavonoid rutin content in the roots, in plants inoculated by *P. chlamydosporia,* may be related to the growth promotion. Otherwise, phytohormonal profiles indicated that JA regulation might be necessary to establish the beneficial endophytic interaction with tomato. In addition to altering root metabolism, this interaction causes systemic changes, as the leaf metabolome was altered in the presence of the fungus. Finally, the presence of the fungus may be acting directly or indirectly for the signaling leading to the activation of metabolic pathways in the root and shoot of tomato. Thus, these metabolic changes may be important to promote plant growth and disease resistance, as observed in the increase of phenolic compounds, flavonoids and tomatidine.

6. Supplementary material

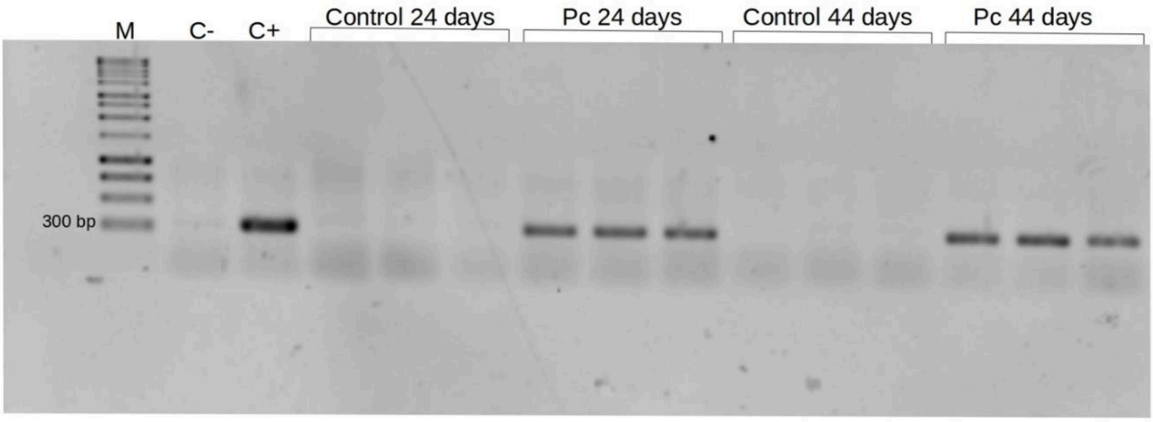

Figure S1: Analysis of the endophytic colonization of tomato roots by *Pochonia chlamydosporia* by PCR. Agarose gel electrophoresis showing detection of a 300 bp amplicon of the VCP1 gene in tomato roots inoculated with P. chlamydosporia (Pc-10) for the treatments Pc 24 days e Pc 44 days. Absence of band for control plants where there was no inoculation of the fungus. Negative control (C–): PCR reaction without DNA; positive control (C+): PCR reaction with DNA from the fungus *P. chlamydosporia* (Pc-10) and molecular marker (M). Amplicon of 300 bp is indicative of the presence of the fungi in root tissues (Lopez-Llorca et al., 2010).

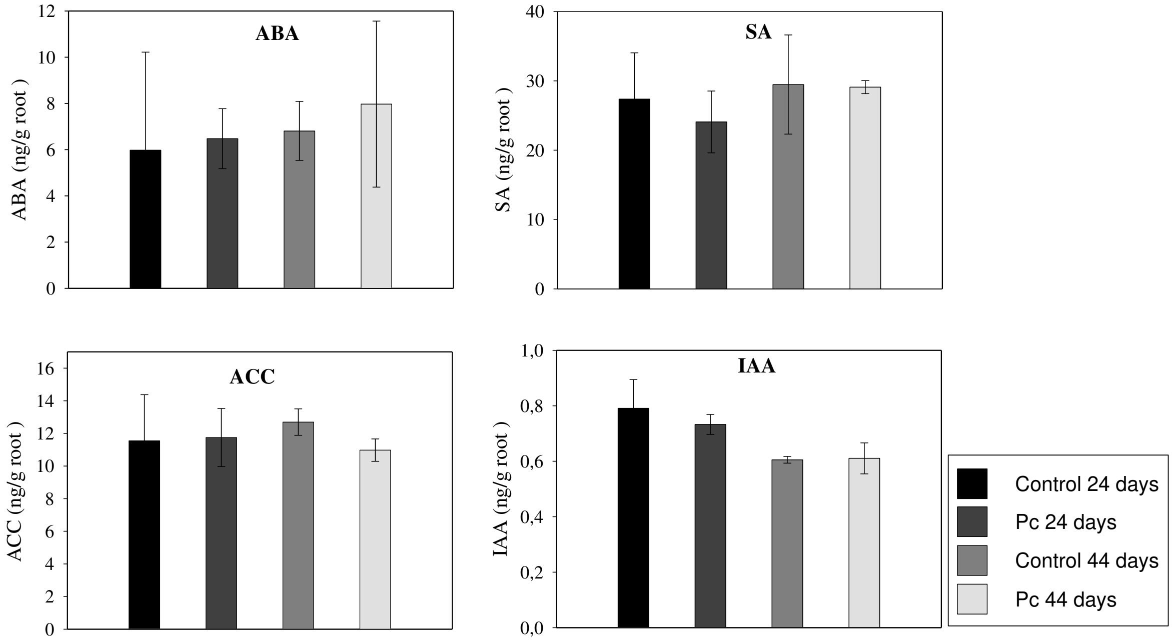

Figure S2: Quantification of phytohormones from tomato roots colonized by *Pochonia chlamydosporia*. Mean followed by only one asterisk differs by the Student's t-test ($p < 0.05$) in the first evaluation period (24 days). Mean followed by double asterisk differs by the Student's t-test ($p < 0.05$) in the second evaluation period (44 days). Pc: *Pochonia chlamydosporia*; ABA: abscisic acid; SA: salicylic acid; ACC: 1-aminocyclopropane-1-carboxylic acid and IAA: indole-3-acetic acid.

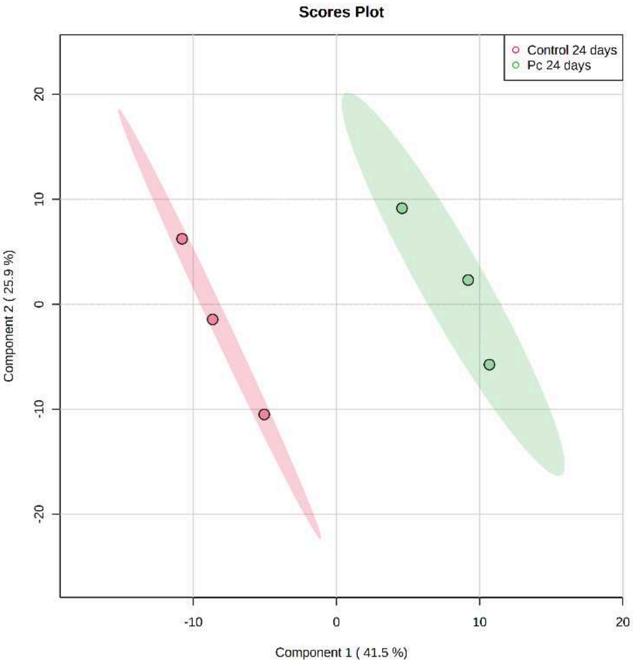

Figure S3: Partial least squares discriminant analysis (PLS-DA) comparison of the LC/MS metabolic profile of tomato root extracts after 24 days of interaction with *Pochonia chlamydosporia* with untreated plants (control).

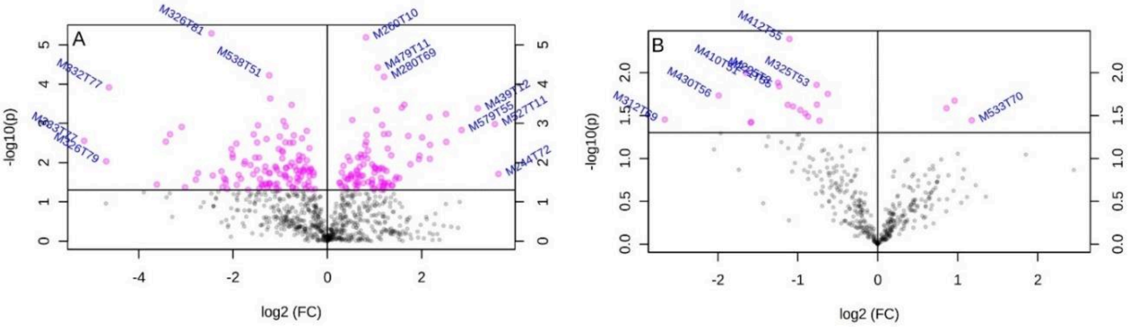

Figure S4: Volcano plot referring to the comparison of metabolites that compose tomato root extracts colonized or not by *Pochonia chlamydosporia.* The gray dot indicates metabolite is not significantly different between treatments. The pink dot indicates a significant difference ($p < 0.05$) for a given metabolite due to the presence of the fungus. Volcano plot **A)** First evaluation period (24 days) and **B)** Second evaluation period (44 days).

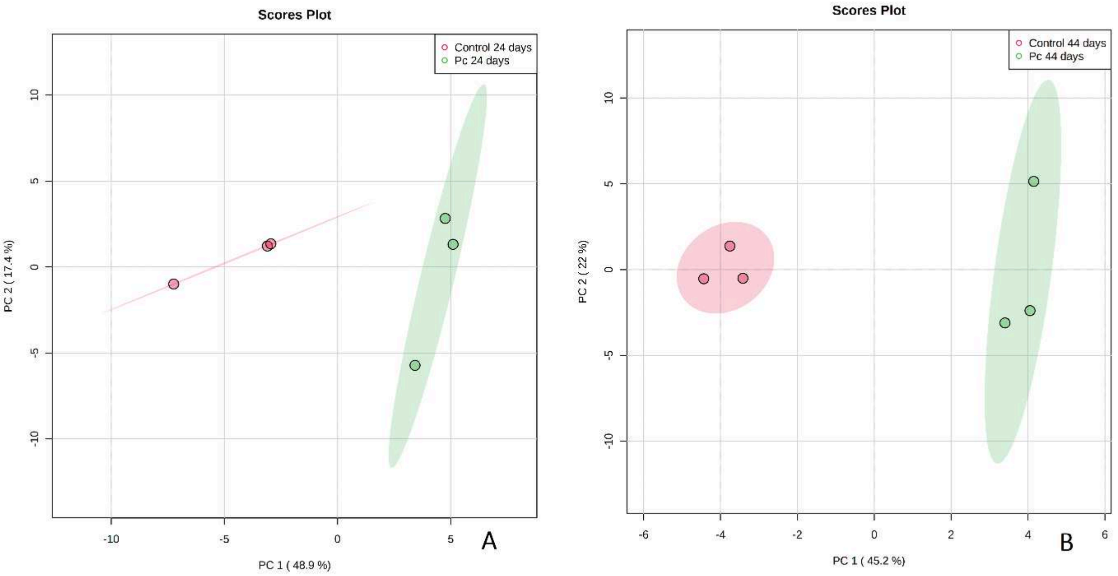

Figure S5: (A) Clustering analysis by Principal component analysis (PCA) in comparing the metabolic profile of LC/MS of a leaf extract from tomato plants not-colonized or colonized by the fungus *Pochonia chlamydosporia* after 24 days. **(B)** PCA in comparing the metabolic profile of LC/MS of a leaf extract from tomato plants not-colonized or colonized by the fungus *P. chlamydosporia* after 44 days.

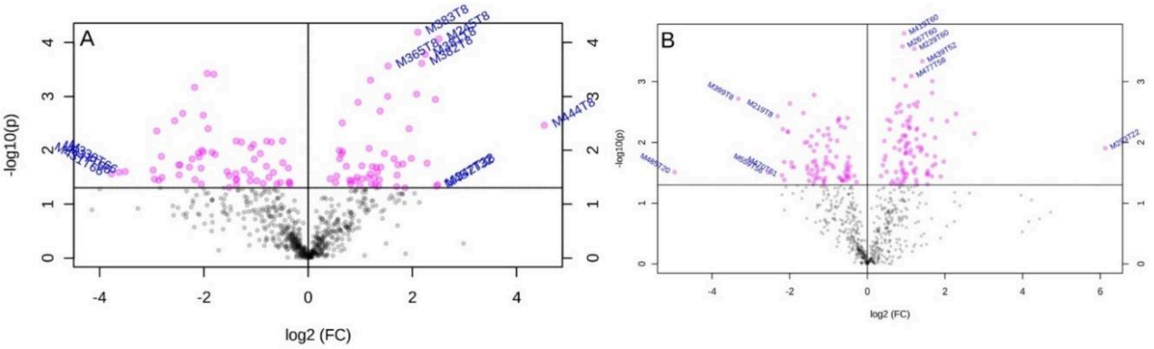

Figure S6: Volcano plot referring to the comparison of metabolites that compose leaf extract from tomato not-colonized or colonized by the fungus *Pochonia chlamydosporia.* The gray dot indicates metabolites that did not show a significant difference between treatments. The pink dot indicates a significant difference ($p < 0.05$) for a given metabolite due to the presence of the fungus. Volcano plot in **(A)** 24 days of colonization and (**B)** 44 days of colonization.

Table S1. Chemical characterization of the soil used in the greenhouse experiment with *Pochonia chlamydosporia*

pH (H_2O)	P (mg/dm^3)	K (cmol$_c$ /dm^3)	Ca^{2+} (cmol$_c$ /dm^3)	Mg^{2+} (cmol$_c$ /dm^3)	Al^{3+} (cmol$_c$ /dm^3)	OM
5.92	2.2	47	0.88	0.26	0	0

OM: organic matter

Table S2. Transition list used as input in the Skyline to the analysis of the phytohormones.

Molecule Name	Precursor Name	Precursor m/z	Precursor Charge	Product m/z	Product charge	Precursor RT (min)	Exact mass
JA	Jasmonic Acid	209	-1	59	-1	9.2	210.12
ABA	Abscisic acid	263.1	-1	153	-1	8.2	264.13
SA	Salicylic acid	136.8	-1	92.9	-1	6	138.03
IAA	Indoleacetic Acid	176	1	129.9	1	7.5	175.06
ACC	ACC	102.1	1	56.2	1	0.6	101.04

Table S3: Transition list used as input in the Skyline to the analysis of the compounds.

Molecule List Name	Precursor m/z	Precursor Charge	Product m/z	Product charge	Precursor RT	Explicit collision energy
4 - hydroxybenzoic acid	139.12	1	121	1	3.85	20
4 - hydroxyflavone	239.07	1	137	1	9.89	20
4-hydroxy-3-methoxy-cinnamaldehyde	179.1	1	147.04	1	7.19	10
7 - hydroxyflavone	239.07	1	137	1	9.89	20
Benzoic acid	123.02	1	77	1	6.82	30
Caffeic acid	179	-1	135	-1	4.61	10
catecol	109.02	-1	91.01	-1	3.52	30
chlorogenic acid	355	1	163	1	3.86	20
coumarin	147.06	1	91	1	7.74	20
curcumin	367.11	-1	173.06	-1	12.46	10
Ferulic acid	193.05	-1	134.1	-1	6.31	20

Isoferulic	193	-1	134	-1	6.31	20
Neochlorogenic acid	353.1	-1	179	-1	3.05	10
N-propil galato	213	1	153	1	7.49	20
p-coumaric acid	163.04	-1	119	-1	5.81	10
sinapic acid	223	-1	164.1	-1	6.34	10
sinapyl alcohol	211	1	133	1	13.91	20
syringic acid	197	-1	121.2	-1	4.7	10
Trans-cinnamic acid	147	-1	77	-1	8.78	30
vanillin	151	-1	92	-1	5.59	20
3.5-dihydroxy benzoic acid	155.02	1	137.01	1	2.65	10
Hesperidin	611	1	303	1	6.8	30
Rutin	611	1	303	1	6.2	30
Naringin	581	1	273	1	6.8	30
Orientin	449	1	329	1	5.8	30
Isoorientin	449	1	299	1	5.6	30
Vitexin	433	1	313	1	6.2	30
Isovitexin	433	1	283	1	6.2	30
Myricetin	319	1	153	1	7.2	30
Morin	303	1	153	1	8	30
Hesperetin	303	1	153	1	6.8	30
Quercitin	303	1	153	1	8	30
Epicatechin	291	1	139	1	5	30
Catechin	291	1	139	1	4.2	30
Kaempferol	287	1	153	1	8.6	30
Luteolin	287	1	153	1	7.9	30
Phloretin	275	1	107	1	7.1	30
Narigenin	273	1	153	1	8.4	30
Genistein	271	1	153	1	8.4	30
Apigenin	271	1	153	1	8.4	30
Daidzein	255	1	137	1	7.7	30
Chalcone	210	1	104	1	11.2	30

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CAPÍTULO 2

RESUMO

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, junho de 2022. **Inoculação de *Pochonia chlamydosporia* desencadeia respostas de defesas em raízes de tomateiro que reduzem a população de *Meloidogyne javanica*.** Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes e Maria Goreti de Almeida Oliveira.

Pochonia chlamydosporia é um fungo de solo utilizado para o controle biológico de nematoides e é o ingrediente ativo em vários bionematicidas comerciais. Este fungo também pode se associar endofíticamente às raízes de várias espécies vegetais, promove o crescimento das plantas e pode induzir resistência sistêmica nas plantas. Além de, parasitar diretamente ovos de nematoides na rizosfera. Neste estudo, diferentes vias e compostos de defesa radicular foram avaliados para identificar mecanismos fornecidos por *P. chlamydosporia* que contribuem para o controle de *Meloidogyne javanica*. A análise ocorreu em dois períodos distintos que foram definidos de acordo com o estágio de desenvolvimento do nematoide. Observou-se que algumas respostas de defesa ativadas pelo fungo são dependentes do ciclo de vida do nematoide. Dentre as análises bioquímicas avaliadas, a colonização radicular de *P. chlamydosporia* proporcionou aumento na concentração de compostos fenólicos, como o ácido clorogênico. Além disso, a expressão de alguns genes avaliados também foi modificada. A interação do fungo com as raízes infectadas por *M. javanica* resultou na maior expressão dos genes PAL (Fenilalanina amônia-liase), LECHS2 (Chalcona sintase) e PI1 (inibidor de protease) no primeiro período avaliado (24 dias). No segundo período (44 dias) houve aumento da expressão devido ao fungo para o gene RBOH. A presença do fungo causou redução na expressão do gene ACC-oxidase e PR-1 (proteínas relacionadas à patogênese 1) nas raízes. Além disso, a inoculação de *P. chlamydosporia* alterou metabólitos e o perfil fito-hormonal da galha formada por *M. javanica* que podem desencadear resposta de defesa na planta envolvendo cascatas de ácido jasmônico e fitosfingosina. Com esta análise, foi possível propor os mecanismos moleculares induzidos pelo fungo que contribuem para o controle de *M. javanica*.

Palavras-chave: Biocontrole. Perfil Metabolômico. Fitohormônios. Flavonoides. Nematoide das Galhas.

ABSTRACT

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, June de 2022. **Inoculation of *Pochonia chlamydosporia* triggers defense responses in tomato roots that reduce the population of *Meloidogyne javanica.*** Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes and Maria Goreti de Almeida Oliveira.

Pochonia chlamydosporia is a soil-dwelling fungus used for the biological control of nematodes and is the active ingredient in several commercial bionematicides. This fungus can also endophytically associate with the roots of several plant species, promotes plant growth and can induce systemic resistance in the plants. In addition to directly parasitizing nematode eggs in the rhizosphere. In this study, different pathways and root defense compounds were evaluated to identify mechanisms provided by *P. chlamydosporia* that contribute to the control of *Meloidogyne javanica*. The analysis occurred in two different periods that were defined according to the development stage of the nematode. It was observed that some defense responses activated by the fungus are dependent on the nematode life cycle. Among the biochemical analysis evaluated, the root colonization of *P. chlamydosporia* provided an increase in the concentration of phenolic compounds, such as chlorogenic acid. In addition, the expression of some genes evaluated was also modified. The interaction of the fungus with the roots infected by *M. javanica* resulted in the highest expression of PAL (Phenylalanine ammonia-lyase), LECHS2 (Chalcone synthase), and PI1 (protease inhibitor) genes in the first period evaluated (24 days). In the second period (44 days) there was an increase in expression due to the fungus for the RBOH (Respiratory Burst Oxidase Homolog) gene. The presence of the fungus caused a reduction in the expression of the ACC-oxidase and PR-1 (pathogenesis-related proteins 1) gene in the roots. Moreover, *P. chlamydosporia* inoculation has changed the metabolite, and phytohormonal profiles of the gall formed by *M. javanica* may trigger plant defense response involving jasmonic acid and phytosphingosine cascades. With this analysis, it was possible to propose the molecular mechanisms induced by the fungus that contribute to the control of *M. javanica.*

Keywords: Biocontrol. Metabolomic Profile. Phytohormones. Flavonoids. Root Knot Nematodes.

1. Introduction

Different pathogens cause plant diseases, which result in reduced productivity of many crops. Among plant pathogens, nematodes stand out, with the genus *Meloidogyne* being one of the most harmful (Jones et al., 2019). *Meloidogyne incognita*, *M. javanica*, and *M. hapla* cause severe losses due to parasitism of a wide range of hosts, wide distribution, and high aggressiveness (Gokte-Narkhedkar et al., 2022; Moens et al., 2017; Moens et al., 2009).

In the host, the *Meloidogyne* genus induces the formation of a specialized structure to obtain its nutrients through the activation of the cell cycle of some vascular parenchyma cells, transforming the host cells into nursing cells. However, cytokinesis, the final phase of mitosis, does not occur, causing an increase in the number of nuclei forming polyploid cells (Almeida et al., 1999). This change in the cycle results in a more metabolically active cell and an accumulation of nutrients occurs to maintain the development of the nematode, which causes an osmotic imbalance (Almeida et al., 1999). To restore balance, water enters the cell, resulting in larger cells, which are called giant cells, and will be used to develop the nematode (Almeida et al., 1999). Simultaneously, the cells close to the nematode and the giant cells multiply, resulting in the formation of galls (Almeida et al., 1999). The formation of this feeding site involves changes in primary and secondary metabolism and cellular modifications in the host that allow the development of J2 into adulthood (Baldacci-Cresp et al., 2015).

Due to its high reproductive capacity, the eradication of these phytopathogens is practically impossible, so for the control of the disease, the best option is the integrated management that involves crop rotation techniques, resistant cultivars, chemical nematicides, and biological control (Sasanelli et al., 2021).

The growing numbers of registration of biological products based on microorganisms such as *Bacillus firmus, Bacillus amyloliquefaciens, Purpureocillium lilacinum, Pochonia chlamydosporia,* and *Trichoderma harzianum* represent the importance that this form of management has assumed in the agricultural scenario. In addition to the efficiency and less contamination of the environment, they are safer for the farmer's health (Lahlali et al., 2022).

In this context, the fungus *P. chlamydosporia* is widely used to control different genera of nematode parasite plant, such as *Globodera, Heterodera, Meloidogyne, Rotylenchulus,* and *Nacobbus* (Manzanilla-López et al., 2017). The direct control mechanism described for this fungus is the adhesion of the hyphae to the eggs and the secretion of enzymes (proteases-EC

3.4, chitinases- EC 3.2.1.14, and lipases- EC 3.1.1.3) that act on the eggshell, allowing the microorganism access to the interior of the eggs (Esteves et al., 2009). Indirectly, this fungus can also contribute to controlling root-knot nematode (RKN) by inducing resistance in plants (Medeiros et al., 2015).

Metabolic alterations resulting from *P. chlamydosporia* colonization of roots may be related to the efficiency of the fungus in controlling nematodes (Gouveia et al., 2022). Therefore, in this study we investigated changes in phenolic compounds, flavonoids, and the expression of genes essential for the defense system in the presence of the fungus in roots infected by *M. javanica*. In addition, metabolomics was used to investigate the effects of the fungus *P. chlamydosporia* on the metabolites that constitute the gall region formed by *M. javanica* and the systemic effects of this interaction on leaves. The understanding of this plant-nematode-fungus interaction, mainly about the manipulation of the host during the development of the disease, may lead to the discovery of new targets and strategies to control root-knot nematodes.

2. Methodology

2.1 Preparation of the fungus, nematodes, and plants

The fungus *P. chlamydosporia* (isolate Pc-10) belongs to the collection of the Laboratory of Biological Control of Phytonematodes of the Federal University of Viçosa, Minas Gerais, Brazil. This fungus was cultivated in autoclaved rice, and the growth was maintained for 21 days at 27 ºC to obtain chlamydospores. After this time interval, these structures were recovered with aqueous extraction. The suspension was quantified using a Neubauer chamber.

The inoculum of *M. javanica*, obtained from the pure population, was multiplied in tomato plants (Santa Clara) in a greenhouse. Eggs were extracted from the roots using the technique described by Boneti and Ferraz (1981). Santa Clara tomato (*Solanum lycopersicum* L.) seedlings (21 days) were transplanted into 300 mL pots containing sterilized soil composed of C horizon clay soil and washed sand in the proportion of 1:1 (V:V).

2.2 Assay in a growth chamber

The interaction of *P. chlamydosporia* with nematode-infected roots was evaluated using the following treatments: *M. javanica* and *M. javanica* + *P. chlamydosporia* (Pc-10). The concentration of the fungus used was 5,000 chlamydospores g^{-1} of soil and for the nematode was 1,000 *M. javanica* eggs per pot (Balbino et al., 2021). First, the fungus was added to the soil containing the tomato seedling and after 5 days the eggs were added to the soil. The experiments were kept at 25° C. The evaluation of the experiment was carried out at 24 and 44 days after transplanting. Collected roots, galls, and leaves were immediately frozen in liquid nitrogen and stored in a -80 ºC freezer for further biochemical analysis. The experimental design was randomized and three biological replicates were used, each replicate consisting of a pool of three plants (nine plants by treatment).

2.3 Biological control of *Meloidogyne javanica* in the greenhouse

Ten plants referring to the second period of analysis (44 days) was used to evaluate the biological control performed by *P. chlamydosporia*, counting the number of eggs and the number of galls per plant. Eggs were extracted from the roots using the technique described by Boneti and Ferraz (1981). The quantification was performed under an optical microscope.

2.4 Quantification of phytohormones (galls and root) by UHPLC-MS QqQ

The extraction of phytohormones from galls (50mg) and roots (100mg) was performed according to the method of Vital (2019). First, the metabolites were separated by ultra-performance chromatography of the UHPLC type (Agilent), using the C18 column (50 mm x 1.0 mm ID, 1.7 μm particle, and 300 A), coupled online to the mass spectrometer Triple Quadrupole (QqQ). The mass spectrometer was used alternating the positive and negative mode and the sample was scanned in the MRM mode (multiple reaction monitoring) to detect each phytohormone (ethylene precursor, 1-aminocyclopropane-1-carboxylic acid (ACC), abscisic acid (ABA), indolyl-3-acetic acid (IAA), salicylic acid (SA), jasmonic acid (JA) (**Table S1**). The data were analysed using the Skyline software and, after obtaining peak area values, absolute quantification (ng/g of tissue) was performed using the standard curve of each hormone.

2.5 Biochemical analysis total root

2.5.1 Total phenolic compounds

The extraction of total phenolic compounds was performed with methanol 80% (v/v) as solvent in 100 mg of root collected from each treatment (*M. javanica* and *M. javanica* + *P. chlamydosporia*). The tubes were kept under agitation for 12 h. The recovered supernatant was used in order to analyse the total phenolic compounds by the Folin-Ciocalteu method (Makkar et al., 1993). The reaction consisted of 60 µl of the supernatants, 60 µl Folin-Ciocalteu reagent 0.25N, and 60 µl sodium carbonate solution 7.5 % (w/v) and incubation for 20 min. Afterward, the absorbance was measured using a UV Spectrophotometer (Shimazu, UV-1800) at 760 nm against a blank without extract. The results were quantified from a standard curve of gallic acid (mg/L), and the result was expressed in gallic acid equivalents in milligrams per fresh root (mg GAE/g).

2.5.2 Evaluation of flavonoids and phenolic compounds by LC/MS

For the extraction of the flavonoids and phenolic compounds, 200 mg of root were used, plus 400 µL of extraction solution (methanol, isopropanol, and acetic acid solution (20: 79: 1), and the extraction method was performed according to Vital (2019). The metabolites were separated by ultra-performance chromatography of the UHPLC type (Agilent), using a C18 column (50 mm x 1.0 mm ID, 1.7 µm particle, and 300 A), coupled online to a triple quadrupole (QqQ) mass spectrometer. The equipment was operated in MRM mode, monitoring flavonoids and phenolic compounds (**Table S2**). The retention times and MRM transitions (multiple reaction monitoring) generated for each standard were tabulated to form the transition list that was used as input to the Skyline, allowing for quantitative analysis of specific compounds in the root.

2.5.3 Lignin

The pellet obtained from the methanol extraction (section 2.5.1) was used to determine the lignin content using the thioglycolic acid method (Campbell et al., 1992). This pellet was washed with water for 2x and after centrifugation, it was dried at 65ºC for 12h. Afterward, the pellet mass was measured, and 1.5mL of thioglycolic acid-HCl 2M mixture (1:10) was added to the flasks and was heat-treated at 90◦C for 4 h. After centrifugation, the supernatant was discarded and 1 mL of distilled water was added as a wash. The material was centrifuged again and the supernatant was discarded. To the pellet was added 1.5 mL of NaOH 0.5M, and the mixture was kept stirring overnight. After centrifugation, 1.5 mL of the supernatant was transferred to a new tube, and 200 µL of concentrated HCl was added and mixed by inversion. The material was kept on ice for 4h. After centrifugation, the pellet was recovered, and 2 mL of NaOH 0.5M was added for dissolution. The samples were diluted with NaOH 0.5M to measure the absorbance at 280 nm. Lignin was quantified as absorbance values per gram dry weight (A280nm g^{-1}).

2.5.4 RNA extraction, cDNA synthesis and expression analysis by qRT-PCR

Plant samples (100 mg from the fresh root) collected for each treatment (*M. javanica* and *M. javanica + P. chlamydosporia*) were used for RNA extraction using Trizol reagent (Invitrogen) according to the manufacturer's instructions. A total of 1 µg of RNA was used for cDNA synthesis with the iScript cDNA Synthesis kit (BioRad) following the manufacturer's instructions. Gene expression was evaluated using an ABI 7500 fast thermal cycler (Applied Biosystems, Foster City, CA, USA) and Fast Master SYBR Green Master Mix (Thermo Fisher Scientific). The amplification reactions were performed with the cycling conditions: 15 s at 95 ° C, 40 cycles at 95 ° C for 3 s; 30 s at 60 ° C and final denaturation at 95 ° C for 20 s. Gene-specific qRT-PCR primers were designed based on their cDNA sequences (**Table S3**). Three technical replicates were performed for each one of the three biological replicates. Gene expression was quantified using the ΔCT method, and the expression levels were calculated as $2^{-\Delta CT}$ (Livak, 2001).

2.6 Untargeted Metabolic profile (galls and leaf) by LC/MS

The extractions of the metabolites from the galls (50mg) and leaves (100mg) were carried out according to Vital et al. (2019). Initially, the separation of the compounds from the extracts occurred by Nano Liquid Chromatography-Mass Spectrometry using the nanoACQUITY UPLC system (Waters, Milford, MA, USA). A trap column and ProteCol GHQ303 C18 capillary column 3.0 µm - 300 µm × 150 mm which operates at a flow rate of 5.0 µL.min-1. The nanoACQUITY UPLC is coupled to the microTOF QII mass spectrometer (Bruker Daltonics, Bremen, Germany) where the metabolites were automatically injected and microESI ionization was used. The mobile phase consisted of (A) water and 0.02% acetic acid (v/v) and (B) acetonitrile and 0.02% acetic acid (v/v), establishing gradients along the run.

The positive mode was used for ion scanning. For MS1 spectra the mass range was established between 100 -1000 m/z, and between 50-1000 m/z for MS2 spectra. Hystar software, version 3.2 (Bruker Daltonics, Bremen, Germany) was used for data analysis, and the spectra were processed using the Data Analysis 4.0 program (Bruker Daltonics, Bremen, Germany). MS2 spectra were obtained for the most intense ions found during the scan and with the mass spectrometer operating in Auto-MS mode. The Proteowizard tool was used to convert the data to mzXML format.

The XCMS platform (https://xcmsonline.scripps.edu) was used to compare leaf and gall extracts' LC/MS profile, and the default settings were used based on Gouveia et al., 2019. The XCMS platform generated a table of metabolites containing the intensity of the detected ions used in the statistical analysis in MetaboAnalyst (http://www.metaboanalyst.ca/) where the data were submitted to logarithmic transformation. The fragmentation list was generated in the generic mgf format by the Data Analysis program and used in the NIST database to identify the ions. Finally, the specters were compared with the database based on fragmentation and precursor m/z value.

2.7 Statistical analysis

The means and standard deviations of the results were calculated. The statistical analysis between the two means was performed using the Student's test, with a significance level of 5%, using the R program (R version 3.6.0). When indicated, the data were subjected to analysis of variance (ANOVA) and Tukey's test with a significance of 5%, using the R program (R version 3.6.0).

3. Results

3.1 Biological control of *Meloidogyne javanica* in the greenhouse

The multitrophic behavior of *P. chamydosporia* interacting with tomato root resulted in the control of *M. javanica* infection. At 24 days after transplanting, when the galls were still small, it was possible to determine that the presence of the fungus reduced the number of galls by 37.18 % when compared to the control treatment (**Figure 1A**). At 44 days after transplanting, it was observed a reduction of 29.47 % in the number of mature galls, which were showing nematode egg masses on their surface (**Figure 1A**). Furthermore, the fungal promoted a reduction of 47% of the number of total eggs in comparison with non-inoculated plants from the control treatment (**Figure 1B**).

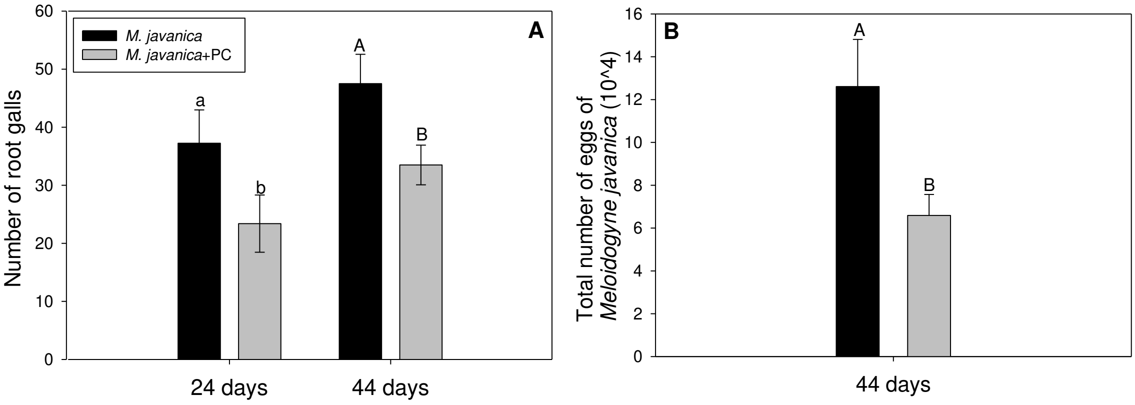

Figure 1: **A)** Number of galls in the presence and absence of *Pochonia chlamydosporia* at 24 days and 44 days after soil infestation with *Meloidogyne javanica*. **B)** Number of eggs 44 days after application *P. chlamydosporia* in soil inoculated with 1,000 eggs of *M. javanica* per pot. According to the T-test, different letters indicate significant differences between treatments ($p < 0.05$). Lower case for the first evaluation period (24 days) and capital letters for the second evaluation period (44 days). Bars represent the standard deviation (n =10).

3.2 Hormonal analysis in the root and gall

Nematodes can induce specific changes in phytohormones to modulate plant defense Thus, the effects of the fungal inoculation on the phytohormonal levels from the plants infected by *M. javanica* were also evaluated in galls and roots. The evaluation of galls from the root with *P. chlamydosporia* and *M. javanica* after 24 days showed a reduction of jasmonic acid (JA) in relation to galls from plants treated only nematode (**Figure 2A**). However, at the end of the cycle (44 days) presence of the fungus increased JA concentration in the gall tissues (**Figure 2A**). The fungus contributed to the increase of JA in the first evaluation period in the roots (but not in the galls tissues) infected by *M. javanica* (**Figure 2A**). For salicylic acid (SA), there was only a change in the roots referring to the second time, which fungus contributed to reducing this hormone (**Figure 2B**). In the second evaluation period of ACC, the fungus caused opposite effects in galls and roots. In galls, the fungus contributed to increasing this intermediate of ethylene synthesis. While in the roots, there was a reduction of ACC due to the action of the fungus (**Figure 2C**).

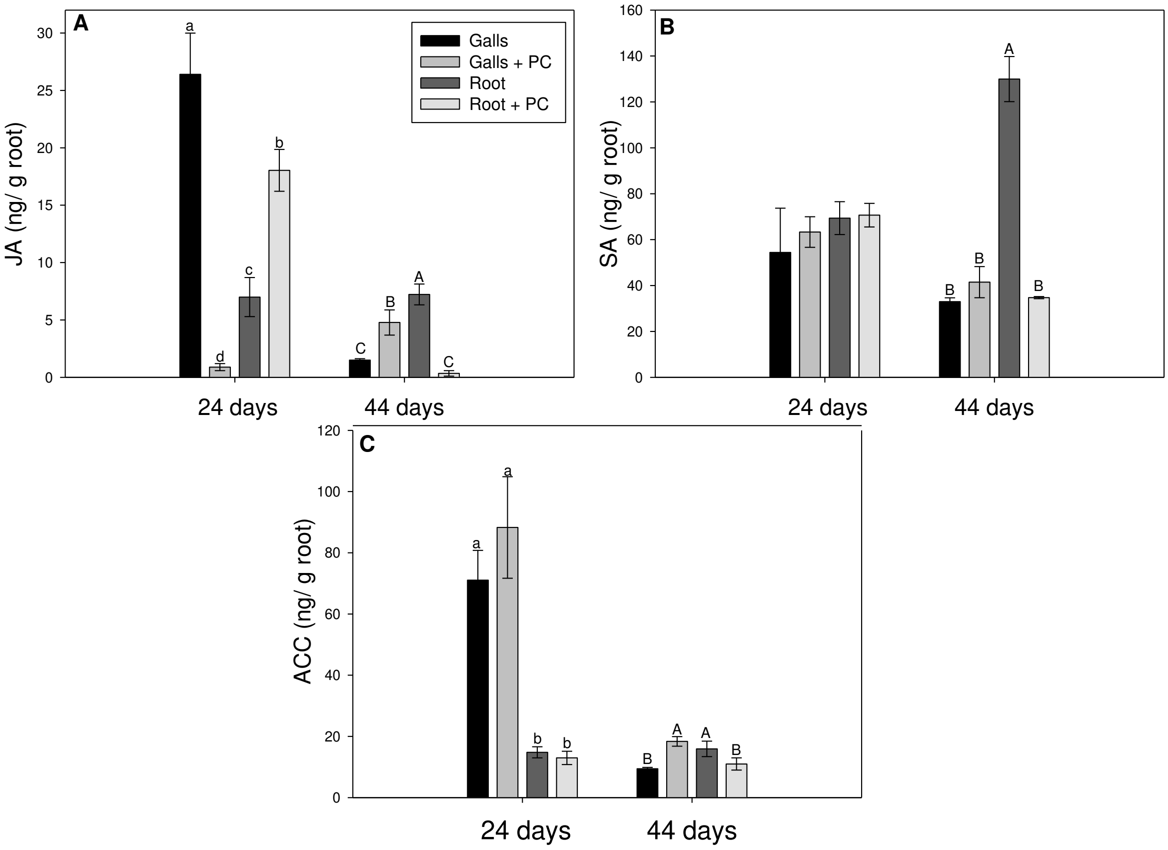

Figure 2: Quantification of phytohormones in galls and roots infected by *M. javanica* and inoculated or not with fungus *Pochonia chlamydosporia*. Quantification of **A)** jasmonic acid (JA); **B)** salicylic acid (SA); **C)** 1-aminocyclopropane-1-carboxylic acid (ACC). According to the Tukey test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$). Lowercase letters refer to the 24 days analysis and capital letters for the 44 days analysis. Bars represent the standard deviation (n=3). PC= *Pochonia chlamydosporia.*

3.3 Biochemical Analysis of the Whole Root

3.3.1 Total phenolic compounds

Roots from tomato plants infected by *M. javanica* and inoculated or not with the fungus *P. chlamydosporia* were submitted to some biochemical analysis to identify changes caused by the fungus that helps in the control of root-knot nematodes. Analysis of total phenolic compounds showed that the amount of these compounds varied with the developmental stages of the nematode, as well as by the presence of the fungus (**Figure 3**). In the early stage of the galls (24 days after inoculation) the presence of the fungus stimulated an increase in the concentration of phenolic compounds. However, at 44 days of interaction, there was a reduction in the concentration of phenolic compounds in the roots with the fungus when compared with the roots only infected by the nematode (**Figure 3A**).

Some phenolic compounds were evaluated in the root extracts and showed alterations according to the nematode development period. After 24 days, it was possible to observe reduced caffeic acid levels by fungal infection (**Figure 3B**). Otherwise, for 4-hydroxy-3-methoxy-cinnamaldehyde, there was a significant difference only in the second evaluation period, in which *P. chlamydosporia* co-infection promoted an increase of this compound (**Figure 3C**). In addition, different behaviors were identified for chlorogenic acid in the presence of the fungus. In the initial phase of the galls (24 days), the fungus increased chlorogenic acid, and in the reproduction phase (44 days), the presence of the fungus reduced this compound in the roots (**Figure 3D**). However, it can highlight an increase in the levels of rutin in plants under fungal infection for both evaluation times (**Figure 3E**).

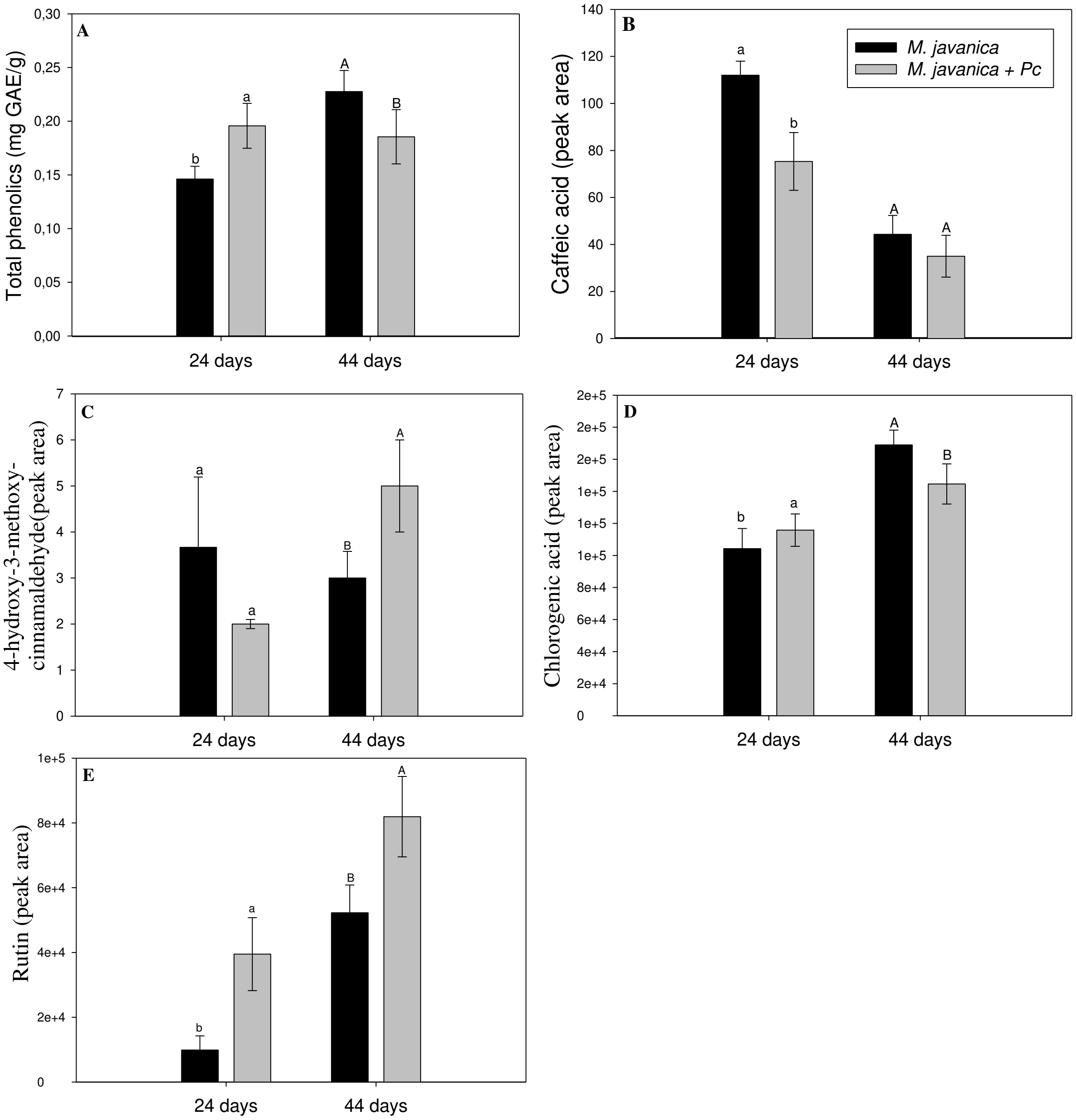

Figure 3: **A)** Total phenolic compounds and relative abundance of phenolic compounds in tomato roots infected by *Meloidogyne javanica* and inoculated or not with the fungus *Pochonia chlamydosporia*. **B)** Caffeic acid; **C)** 4-hydroxy-3-methoxy-cinnamaldehyde (Coniferaldehyde); **D)** Chlorogenic acid. **E)** Rutin. According to the T-test, different letters indicate significant differences between treatments ($p < 0.05$). Lower case for the first evaluation period (24 days) and capital letters for the second evaluation period (44 days). Bars represent the standard deviation (n=3).

3.3.2 Lignin

Lignin was quantified due to its importance as a physical barrier in the root cells against nematode penetration. In the first evaluation period, there was less lignin deposition in the roots from plants that contained the fungus compared to the roots without the fungus (**Figure 4**). However, in the second evaluation period, the fungus did not change the lignin content from the roots infected by the nematode (**Figure 4**).

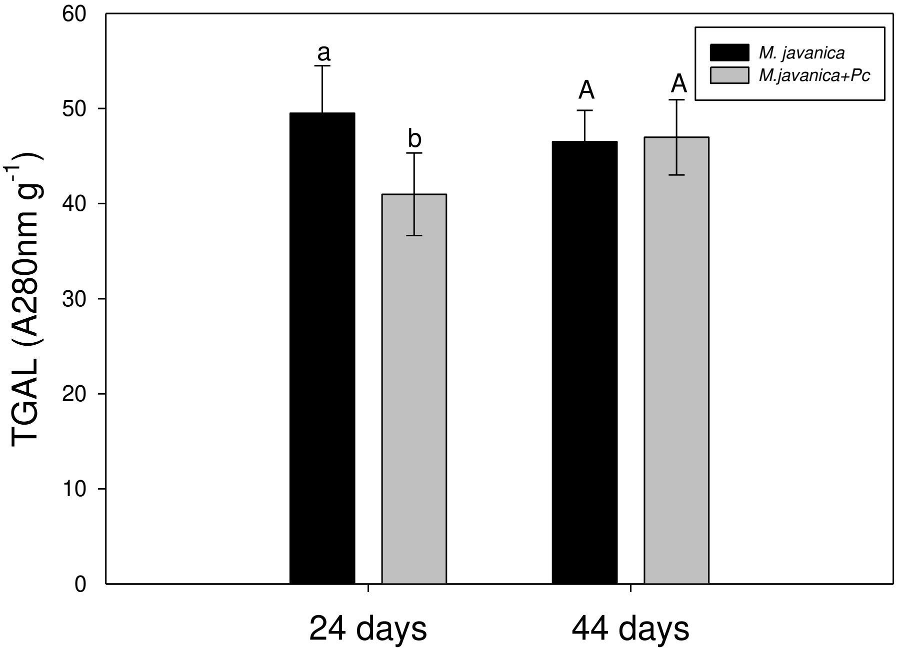

Figure 4: Quantification of lignin-thioglycolic acid (LTGA) derivatives in tomato roots infected by *Meloidogyne javanica* and inoculated or not with fungus *Pochonia chlamydosporia*. According to the T-test, different letters indicate a significant difference between treatments ($p < 0.05$). Lower case for the first evaluation period (24 days) and capital letters for the second evaluation period (44 days). Bars represent the standard deviation (n=3).

3.3.3 Gene expression

Fungal co-inoculation promoted alterations in the expression of some genes involved in the defense against *M. javanica* in tomato roots. In the first evaluation period (24 days), the presence of the fungus in infected roots by *M. javanica* contributed to a higher expression of PAL (Phenylalanine ammonia-lyase), LECHS2 (Chalcone synthase), and PI1 (protease inhibitor) genes (**Figure 5A, 5B, and 5E**). There was an increase in expression in the second period due to the fungus only for the RBOH (Respiratory Burst Oxidase Homolog) gene (**Figure 5C**). For the PR1 gene (Pathogenesis-Related), there was a reduction in expression for both periods evaluated (**Figure 5D**). The presence of the fungus also caused a reduction in the expression of the ACC-oxidase gene in the second evaluation period (**Figure 5F**).

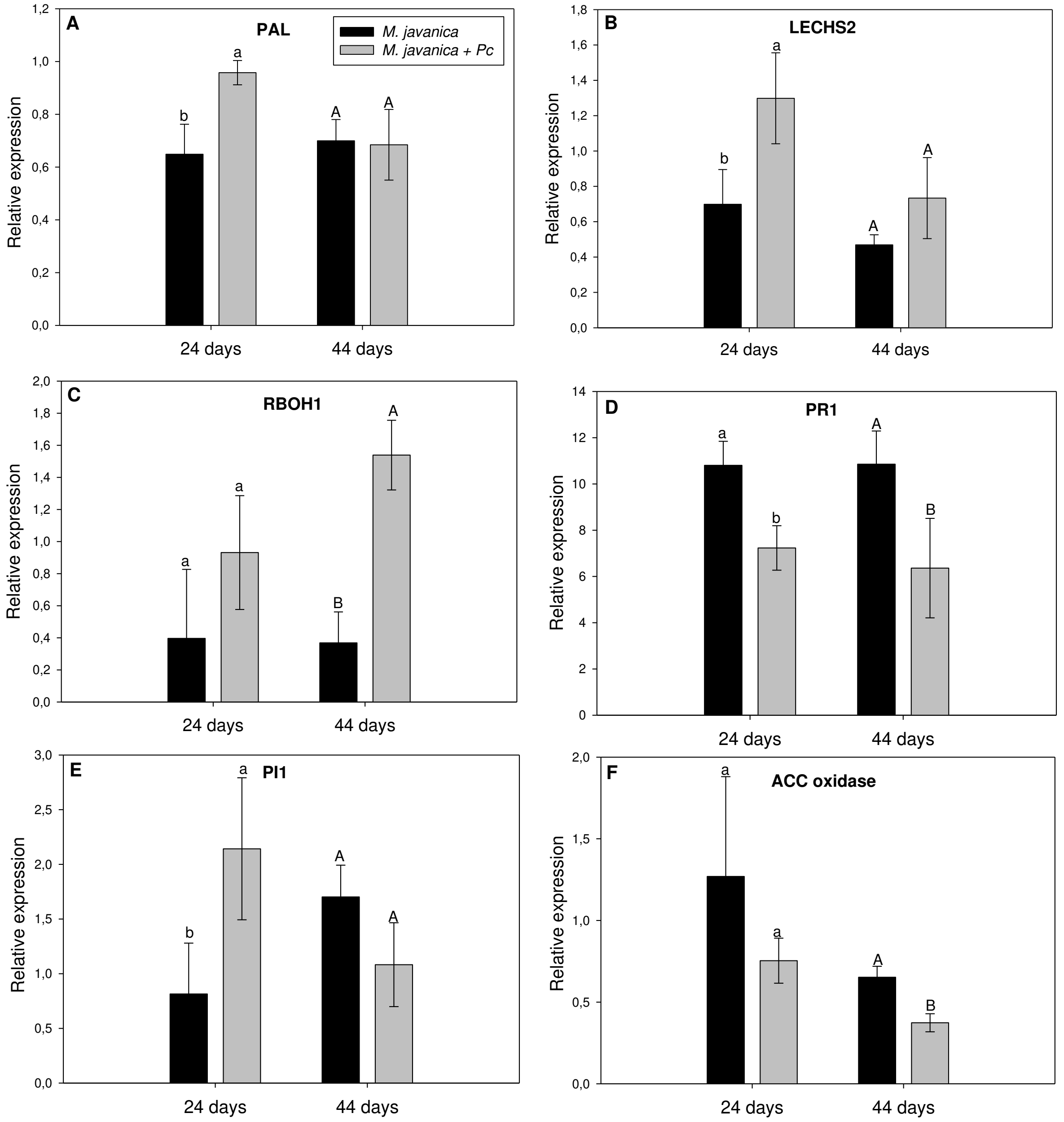

Figure 5: Gene expression analysis by qRT-PCR from tomato roots infected by *Meloidogyne javanica* in the presence or absence of the fungus *Pochonia chlamydosporia*. In **(A)** PAL, Phenylalanine ammonia-lyase; (**B**) LECHS2, (**C**) RBOH, **D)** PR1, **E)** PI1 and **F)** ACC-oxidase. The actin gene was used as endogenous gene. Expression levels were calculated as $2^{-\Delta CT}$ values to compare the treatments' relative expression. According to the T-test, different letters indicate a significant difference between treatments ($p < 0.05$). Lower case for the first evaluation period

(24 days) and capital letters for the second evaluation period (44 days). Bars represent the standard deviation (n=3).

3.4 Evaluation of metabolites in galls

As the presence of *P. chlamydosporia* caused alterations in phenolic compounds, flavonoids, and in some genes in the roots infected by *M. javanica*, we also evaluated untargeted metabolic profiles by LC/MS of the gall region, an essential structure in the maintenance of the nematode life cycle.

Thus, LC/MS profiles from plants infected by *M. javanica* in the presence or absence of fungus were compared to verify changes in the gall region. The principal component analysis (PCA) was used for visualizing the global alteration in the metabolism in response to co-inoculation. The PCA plot of the first-period revealed a separation between the set of gall metabolites from plants non-inoculated and inoculated with *P. chlamydosporia*, with 90.3% of the total variance observed, where the main components PC1 and PC2 explain 62.6 % and 27.7 % of the variance, respectively (**Figure 6A**). The separation between groups indicates that the abundance of metabolites in galls whose roots were colonized by the fungus can be distinguished from galls in which roots were not colonized.

In the reproductive stage of the nematode (44 days), the abundance of gall metabolites was also altered due to *P. chlamydosporia* colonization in the root, as can be seen in PCA, where the first two principal components (PC1 and PC2) explain 57.7 % of the variance of the data (**Figure 6B**).

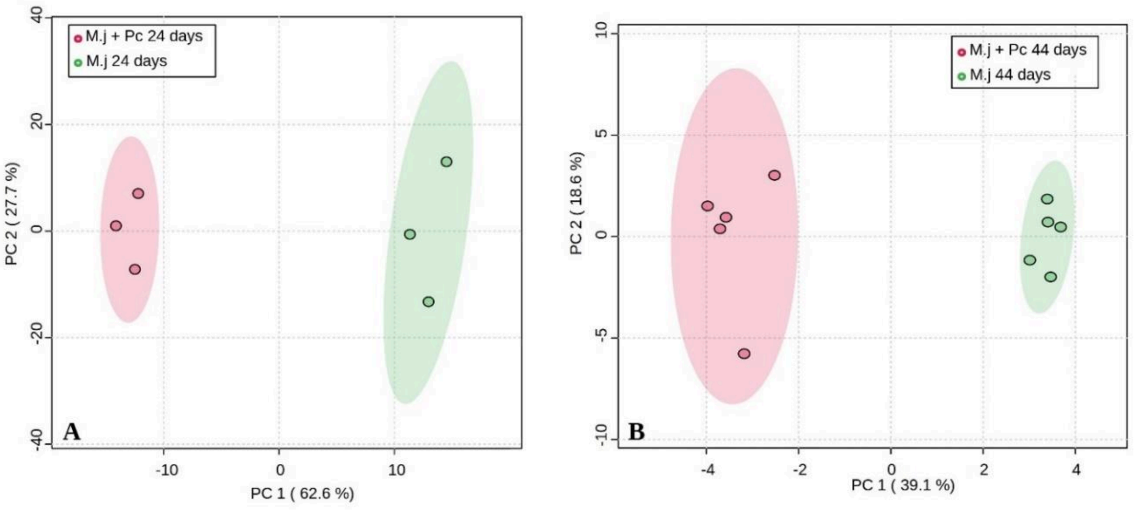

Figure 6: **A)** Principal component analysis (PCA) comparison of the LC/MS metabolic profile of galls from tomato roots infected by *Meloidogyne javanica* and inoculated or not by the

fungus after 24 days. **B)** Principal component analysis (PCA) comparison of the LC/MS metabolic profile of galls from tomato roots infected by *Meloidogyne javanica* and inoculated or not by the fungus after 44 days.

Heatmap cluster analysis was used to indicate galls metabolites with an increase or decrease in abundance due to the presence or absence of the fungus in the root. To verify the metabolites with significant variations in abundance ($p < 0.05$) the T-test was performed and of the 1512 ions detected for the first time (24 days) in the galls, 703 ions showed a statistical difference. The predominance of ions with increased abundance in response to the presence of the fungus stands out, as can be seen in the heatmap (**Figure 7A**). In the second period (44 days) 444 ions were detected and 118 ions showed a significant difference in the presence of the fungus. As in the first period, there was an increase in the abundance of metabolites in the presence of the fungus (**Figure 7B**).

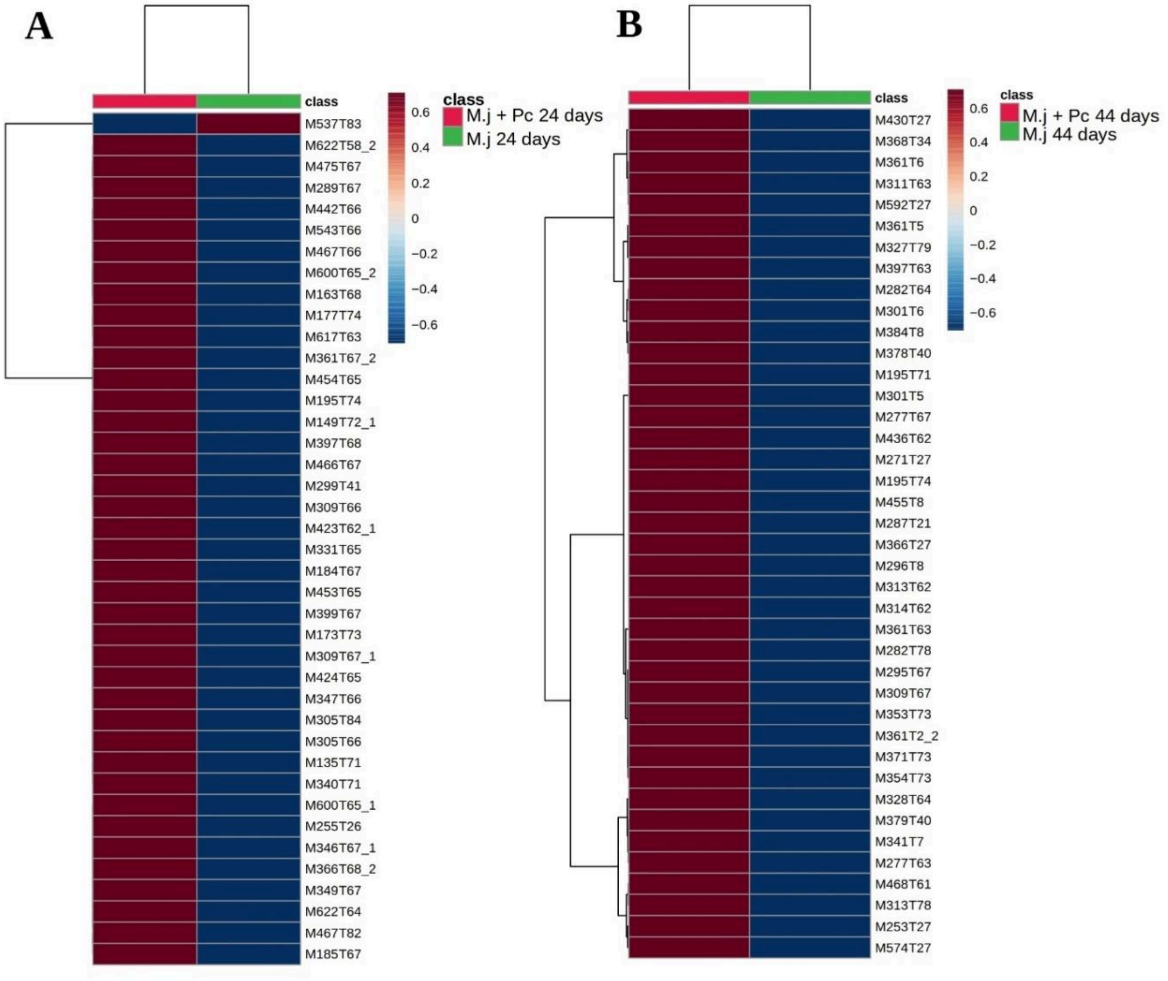

Figure 7: Heatmap with altered ions in galls in the presence and absence of the fungus *Pochonia chlamydosporia* in root **A)** first evaluation period (24 days) and **B)** second evaluation period (44 days).

In order to identify metabolites with statistical significance, fragmentation spectra and precursor mass values (m/z) were used for comparison with the metabolite library NIST. The putative identification of some compounds was possible from the library (**Table 1**).

Table 1: Annotation of compounds present in galls from roots colonized by *Pochonia chlamydosporia*.

Identification	**Fold change***	**Theoretical (m/z)**	**Observed (m/z)**	**p-valor**	**Interaction (days)**
cis-5,8,11,14 Eicosatetraenoic acid	+4.98	305.2475	305.2012	1.33 E-4	24
Epicatechin	+4.66	291.0863	291.1836	2.16 E-4	24
1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	+8.55	454.2928	454.2701	1.7 E-5	24
N-Octadecanoyl-L-homoserine lactone	+5.11	368.3159	368.3435	8.14 E-4	44
1-Palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine	+2.18	454.2928	454.2701	0.0044	44
Phytosphingosine	+1.7	318.3003	318.2821	0.0098	44

* Fold change with positive signal (+) indicates an increase and negative (-) indicates a decrease in the abundance.

3.5 Metabolic alteration in the leaf

The presence of the fungus *P. chlamydosporia* in roots infected by *M. javanica* caused changes in leaf metabolites at different stages of nematode development (24 and 44 days). Principal component analysis (PCA) was used to assess the systemic effect of fungal colonization. In the first evaluation period (24 days), the comparison of metabolic profiles from leaves of plants co-inoculated or not revealed that the replicates of the two treatments were grouped separately with 78.8 % of the total variance between the two groups, and PC1 and PC2 explain 55.5 % and 23.3 % of the variance, respectively (**Figure 8A**). For the second evaluation period, the separation between groups was also identified using the PCA method, in which principal components 1 and 2 explained 70.1 % of the total variation between treatments (**Figure 8B**). The separation between groups indicated that the abundance of leaf metabolites from plants infected with *M. javanica* and cultivated with *P. chlamydosporia* are distinct from plants not colonized by the fungus throughout the nematode's life cycle.

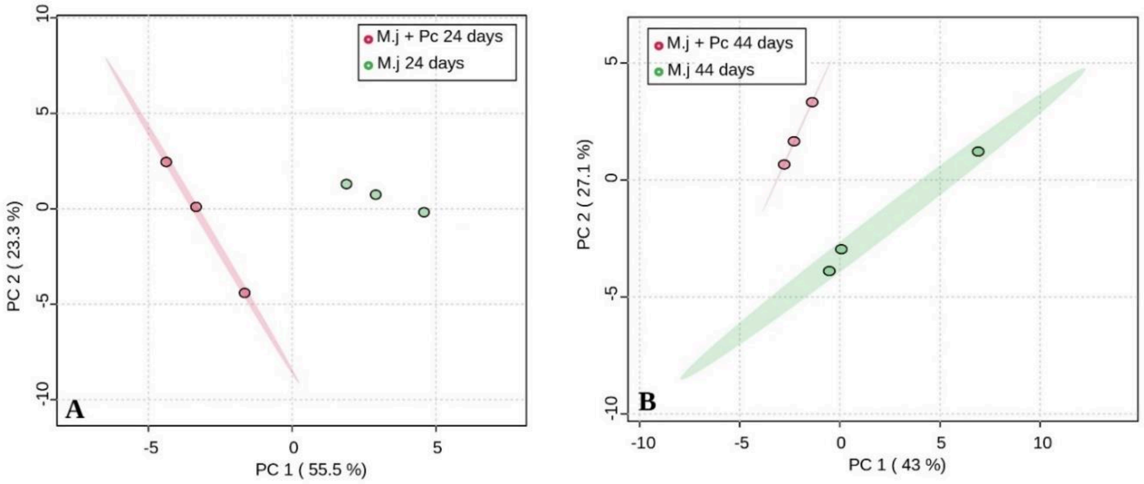

Figure 8: Principal component analysis (PCA) **A)** compares the LC/MS metabolic profile of a leaf extract from tomato plants infected by *Meloidogyne javanica* and colonized or not by the fungus after 24 days. **B)** Comparison of the LC/MS metabolic profile of a leaf extract from tomato plants infected by *Meloidogyne javanica* and colonized or not by the fungus after 44 days.

In the first evaluation period, 620 ions were monitored in the leaves and of these 139 ions showed a statistical difference in the presence of the fungus (p-value <0.05), but only for one ion (M416T40) it was possible to suggest the identification from the comparison with the NIST database. Possibly M416T40 ion corresponds tomatidine due to the similarity of fragmentation with the compound in the database. There was an increase in the abundance of this compound when the fungus colonized tomato root.

The metabolic evaluation of the leaves in the second period remained altered when the fungus was present. Of the 642 monitored ions, 74 showed a statistical difference (p-value <0.05), and by comparison with the database, it was not possible to suggest the identification of these compounds. Tomatidine was altered and identified in this evaluation but did not show the statistical difference as in the first period.

4. Discussion

The fungus *P. chlamydosporia* has been used to control plant-parasitic nematodes mainly by the capabilities of egg parasitism (Manzanilla-López et al., 2014). In addition, this fungus can colonize the roots endophytically (Escudero & Lopez-Llorca, 2012), and it can promote systemic alterations in the metabolism of root and shoot (Gouveia et al., 2022). Thus, the interaction between the fungus and the root infected with nematode can provide biochemical changes that can be important for the control of plant-parasitic nematodes. These modifications have been barely studied, so we evaluated some cascades of plant defense triggered by fungal colonization and its effect in the infection by the root-knot nematode *M. javanica*.

Inoculation of *P. chlamydosporia* to tomato promoted plant growth in the absence of nematode attack (Gouveia et al., 2022), and it was observed that the nematode development was also impaired by fungal colonization of the roots. Thus, the fungal inoculation showed a dual beneficial effect for the plants.

In fact, the phytohormonal profiles from roots infected by *M. javanica* were altered by the presence of fungus. There is much information about this modification's initial phase of nematode infection. However, information is scarcer when this interaction involves a biological control agent for intermediate stages and at the end of the nematode's life cycle. The suppression of defenses through hormonal responses is one of the mechanisms the nematode uses to manipulate the host defense (Gheysen et al., 2019). Thus, phytohormonal changes promoted by fungus may be related to the reduction of galls number in tomato roots colonized by the fungus.

When analyzing galls and non-galled root regions separately, it was possible to observe that the phytohormonal levels were different between these regions. The increase of jasmonic acid in the second evaluation period in the galls due to the presence of the fungus can stimulate transcription factors responsible for the synthesis of protease inhibitors (Koiwa et al., 1997). These can increase the resistance to the nematode of the galls when overexpressed in roots (Urwin et al., 1998). Tomato plants sprayed with jasmonic acid showed higher protease inhibitor (PI2) expression in roots and reduced egg mass (Zhou et al., 2015). In the root, there was the highest amount of jasmonic acid in the presence of the fungus in the first evaluation period. This increase may indicate a defense mechanism provided by the fungus to protect against gall induction in new root regions and may explain the reduction in the galls in the treatment with *P. chlamydosporia* (Chan et al., 2010; Martínez-Medina et al., 2017).

It was observed a significant reduction of the JA levels by the fungal presence in the roots in the reproductive stage of nematode. However, this reduction possibly does not mean that the root is more susceptible to attack by new juveniles from the first cycle since mutant tomato plants with a deficiency in the production of jasmonic acid did not show a greater invasion of *M. incognita* (Martínez-Medina et al., 2017). Furthermore, the penetration was not altered in tomato roots with a higher concentration of phytocystatin (cysteine protease inhibitor) (Chan et al., 2010).

At the end of the nematode cycle (44 days), salicylic acid was reduced in the roots treated with the fungus. This hormone is characteristic in defense responses to biotrophic pathogens, being the subject of studies at the beginning of the interaction with the pathogen. However, its behavior at the end of the cycle is fundamental to understanding the plant's response to new pathogen invasions. Furthermore, this decrease in salicylic acid in the roots may result from the deviation of phenylalanine to the production of other defense compounds since the biosynthesis of phenylpropanoids provides different compounds with nematicidal activity, such as flavonols (Hamamouch & Adil, 2019).

In the second evaluation period, the ethylene hormone precursor, ACC (1-aminocyclopropane-1-carboxylic acid), was detected in greater quantity in galls from plants treated with *P. chlamydosporia*. Greater ethylene production responds to abiotic and biotic stresses that trigger adaptive responses and influence other hormonal signaling pathways. Mutants of *A. thaliana* overexpressing ethylene were less attractive to root-knot nematodes (Fudali et al., 2013). However, there is no information on the role of this hormone in galls to help understand the increase in ACC. The production of ethylene in the galls is proportional to the production of its precursor ACC. In that case, there may be a greater production of hydrogen cyanide, as in the conversion of ACC to ethylene, this compound is released. Furthermore, this compound can generate toxicity to the nematode due to its nematicidal character (Robinson et al., 1986). Some biological control agents for nematodes are chosen based on their ability to produce hydrogen cyanide (Kang et al., 2018; Abd El-Rahman et al., 2019).

The expressions of some genes in the plant defense were evaluated in the tomato roots to investigate regulatory cascades triggered by the fungus presence. *P. chlamydosporia* altered the expression of root genes when the galls were fully formed (24 days) and in the period corresponding to the nematode reproduction (44 days). In the first evaluation period (24 days), the interaction of *P. chlamydosporia* with roots infected by *M. javanica* resulted in increased expression of the PAL gene (**Figure 9**). This gene corresponds to Phenylalanine ammonia-lyase that catalyzes the production of trans-cinnamate, the first and main step of the phenylpropanoid pathway. This pathway results in the production of different compounds (phenols, lignin, flavonoids, and hormones) that can contribute to protecting the plant against nematode attack and development (Chin et al., 2018; Yan et al., 2021).

This increase in the PAL gene expression may have contributed to the higher production of total phenolic compounds in the presence of the fungus detected in the first evaluation period. The increase in the production of phenolic compounds occurs in resistant cultivars of different species (tomato, wheat, barley, rice and banana) as a defense response against root-knot nematodes (Banora et al., 2019; Rezk et al., 1987; Galeng-Lawilao et al., 2018; Wuyts et al., 2007). Thus, the association of *P. chlamydosporia* with the root contributed to the intensification of the defense response, producing a larger level of phenolic compounds that can interfere in the development of the nematode.

As fungus presence promoted a higher production of phenolic compounds, the abundance of some phenolic compounds was also evaluated. The higher level of chlorogenic acid occurred in roots under fungal colonization. This result correlated with the reduction of the tomato root infection by nematode since tomato and soybean resistant genotypes to root-knot nematode has a more significant amount of this compound during nematode infection (Rani et al., 2009; Ramzan et al., 2021). In addition, in plants susceptible to the root-knot nematode, the pathogen induces the reduction of this compound in the roots (Martínez-Medina et al., 2021). As highlighted in Figure 9, in the interaction of the fungus with the root infected by *M. javanica*, there was a more accumulation of chlorogenic acid in the first evaluation period, which indicates a possible mechanism used by *P. chlamydosporia* to help control plant parasitic nematodes.

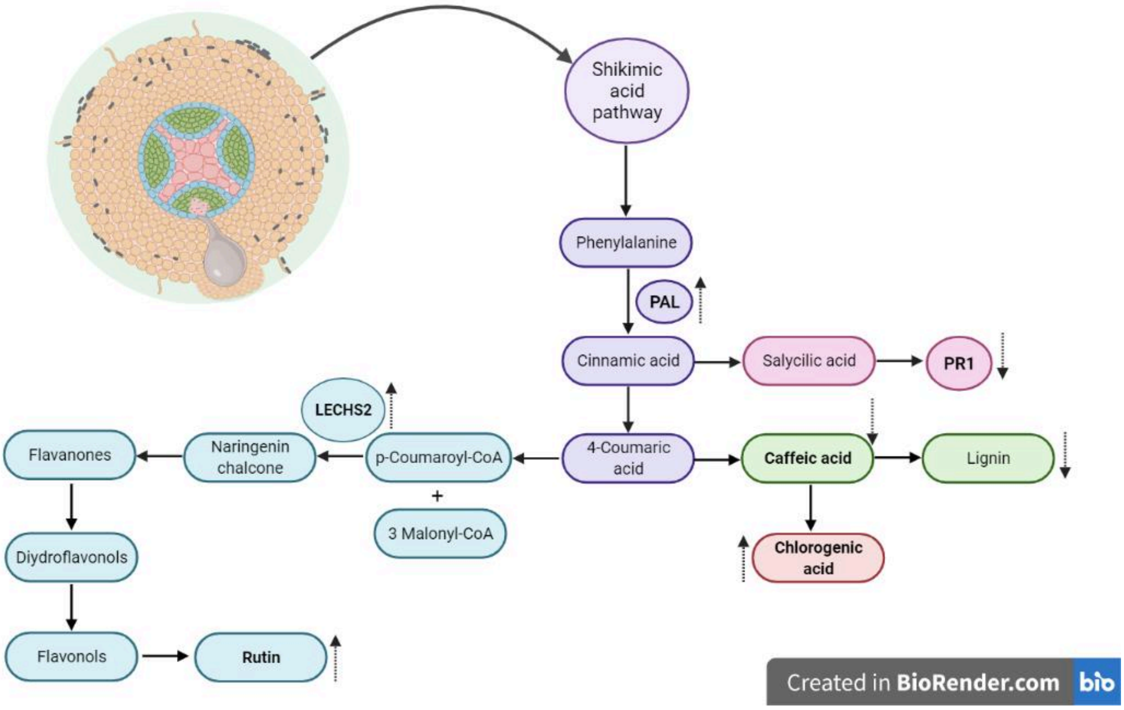

Figure 9: Summary of biochemical changes caused by *Pochonia chlamydosporia* colonization after 24 days in *Meloidogyne javanica* infected root. Compounds in bold correspond to the compounds evaluated in the study. Up arrow indicates compound increase due to fungus presence and down arrow indicates compound decrease due to fungus.

The interaction established between the fungus and the roots infected by *M. javanica* can also cause the reduction of others phenolic compounds, such as caffeic acid (**Figure 9**). This reduction in the first evaluation period may be the result of a deviation of this compound to the production of chlorogenic acid, which is formed from the condensation of caffeic acid to quinic acid (Dixon et al., 1995) since there was an increase in chlorogenic acid in the first evaluation period.

Caffeic acid is also a primary precursor in lignin synthesis, and an increase in its synthesis can result in cell wall reinforcement that restricts root-knot nematode invasion and migration (Sato et al., 2021; Riaz et al., 2018). Thus, the reduction in the first evaluation period detected in the quantification of lignin in the roots colonized by the fungus correlated with to reduction of caffeic acid and increase of chlorogenic acid (**Figure 9**). Furthermore, the period evaluated (24 days) corresponds to the phase of maximum fungal colonization (Escudero & Lopez-Llorca, 2012), so this small reduction in tissue lignification was necessary for fungal growth since high levels of root lignin can constrain fungal colonization, compromising its benefits (Bennet et al. al., 2015). Moreover, changes in the lignin levels are not involved in the mechanism of the reduction of nematode triggered by fungus presence.

In the second evaluation period, the roots with the fungus showed no significant difference in the quantification of lignin in relation to the roots only infected by the nematode, indicating that the reduction found in the first period may have occurred to allow better colonization of the fungus. Furthermore, the increase in the 4-hydroxy-3-methoxy-cinnamaldehyde in the second period (**Figure 10**), under fungal colonization, may have contributed to the formation of lignin, as this compound is a precursor of coniferol, one of the main monolignols constituent of lignin (Sakamoto et al., 2020).

Otherwise, in the first evaluation period, there was an increase in the expression of the LECHS2 in the roots infected by RKN and in the presence of the *P. chlamydosporia* (**Figure 9**). This gene is responsible for synthesizing naringenin chalcone, a precursor of flavonoids that can act as a plant defense response to biotic and abiotic stresses (Chin et al., 2018; Šamec et al., 2021). Thus, the higher expression of this gene may represent a component of the signaling cascades to increase the flavonoids production. The increase in the expression of genes related to flavonoids and its accumulation in the roots have been observed in genotypes resistant to nematode attack (Chin et al., 2018). For root-knot nematodes, it was observed that in plants deficient in flavonoid synthesis, there was no change in the number of formed galls, but the galls were smaller and had a reduced number of pericycle cells (Wasson et al., 2009). *In vitro* tests have indicated that flavonoids may affect the nematodes promoting periods of reversible inactivity, modifying their migration towards the roots, and death (Wuyts et al., 2006). Among the flavonoids, the flavonol class has important compounds in the plant-nematode interaction, such as quercetin, which roots with greater amounts of this compound are less attractive to invasion by *M. incognita* (Kirwa et al., 2018).

Rutin is a glycoconjugate flavonol containing quercetin as aglycone. The fungus presence triggered a signal for increased rutin levels in the RKN infected roots in both the evaluation periods (**Figure 9**). It was characterized as a compound of low nematicidal activity *in vitro* for root-knot nematodes, and there is no study evaluating the accumulation of rutin in roots infected by nematodes (Wuyts et al., 2006; Ntalli et al., 2011). In the literature, this compound is known for its antioxidant activity, which suggests that the increase in this compound provided by the fungus can contribute to attenuating oxidative stress, as in the invasion process of juveniles (Yang et al., 2008). Rutin have been also characterized as agent reducing the insect survival in soybean plants (Gomez et al., 2021).

In this process of invasion, the plant faced with the nematode attack increases the production of reactive species reactive oxygen (ROS) in the apoplast (Melillo et al., 2011). At the same time, the plant activates its antioxidant pathway to protect the plant cell from this oxidative damage (Gillet et al., 2017). This response to the early stages of infection is very well characterized. According, it is possible to suggest a greater production of reactive oxygen species at 44 days due to greater expression of the RBOH1 (RESPIRATORY BURST OXIDASE HOMOLOG) gene in the presence of the fungus (**Figure 10**). The expression of this gene signals ROS induction in the root apoplast, and the role in RKN resistance was confirmed in tomato silenced for RBOH1. In these plants increased susceptibility to RKN in relation to the control, resulting in a greater number of galls (Song et al., 2018). Thus, the increased production of reactive oxygen species may represent another mechanism by which *P. chlamydosporia* controls root-knot nematodes.

In roots colonized by beneficial endophytes, adaptive responses occur to that the interaction occurs asymptomatically (Tan & Zou, 2001). So, it may be necessary to suppress some defense mechanisms for the colonization of the microorganism to occur (Trivedi et al., 2020). In the interaction of *P. chlamydosporia* with tomato roots infected by *M. javanica* in the two evaluation periods, there was the repression of the PR-1 gene (**Figures 9 and 10**). Protein resulting from translation of this gene belongs to the group of pathogenesis-related proteins (PR) that are synthesized to restrict the development and spread of the pathogen in the plant.

This defense mechanism is observed in plants infected by nematodes, which increase the expression of PRs genes (Martınez-Medina et al., 2017; Oliveira et al., 2012). In fact, overexpression of the PR-1 gene in *A. thaliana* resulted in reducing successful *M. incognita* infection (Hamamouch et al., 2011). *Trichoderma harzianum* inoculation in tomato roots infected by *M. incognita* also contributed to the greater expression of PR-1. It may be one of the mechanisms that helped in the control provided by the fungus (Leonetti et al., 2017).

However, our results show that *P. chlamydosporia* contributed to the repression of this gene, indicating possible down-regulation triggered by fungus to maintain its colonization in the roots. This mechanism is adopted by arbuscular mycorrhizal fungi to achieve a compatible interaction and consequently colonize the host (Pozo & Azcón-Aguilar 2007). Though the fungus causes the suppression of an important gene in the defense against nematodes, its use is still advantageous due it caused a significant reduction in the number of eggs at the end of the life cycle because of the various contributions that the fungus provided to the plant.

On the other hand, the presence of *P. chlamydosporia* promoted an up-regulation in the expression of the PI1 gene (serine protease inhibitor) in the first evaluation period (**Figure 10**). Protease inhibitors belong to the family of PR proteins (Pathogenesis Related Protein) and its are very important in the defense system against different pathogens. In tomato plants resistant to *M. incognita* there have been observed a greater expression of the ISP gene (also a serine protease inhibitor). For susceptible plants, activation of this gene was very low after infection by this pathogen (Zinovieva et al., 2021). From the third day of nematode infection, the resistant plant responded with greater expression of the ISP gene and this response increased progressively until the twentieth day after infection (Zinovieva et al., 2021).

The period of evaluation of gene expression in the study by Zinovieva et al., (2021) corresponds to the sedentary period of nematode development, the same period evaluated in our experiment, which indicates that protease inhibitors can act as a possible defense response. Because in this sedentary stage the nematode secretes different proteases with functions in embryogenesis, feeding, and egg maturation (Antonio de Souza Junior et al., 2013). Consequently, the inhibition of these proteases by inhibitors can compromise nematode development and reproduction.

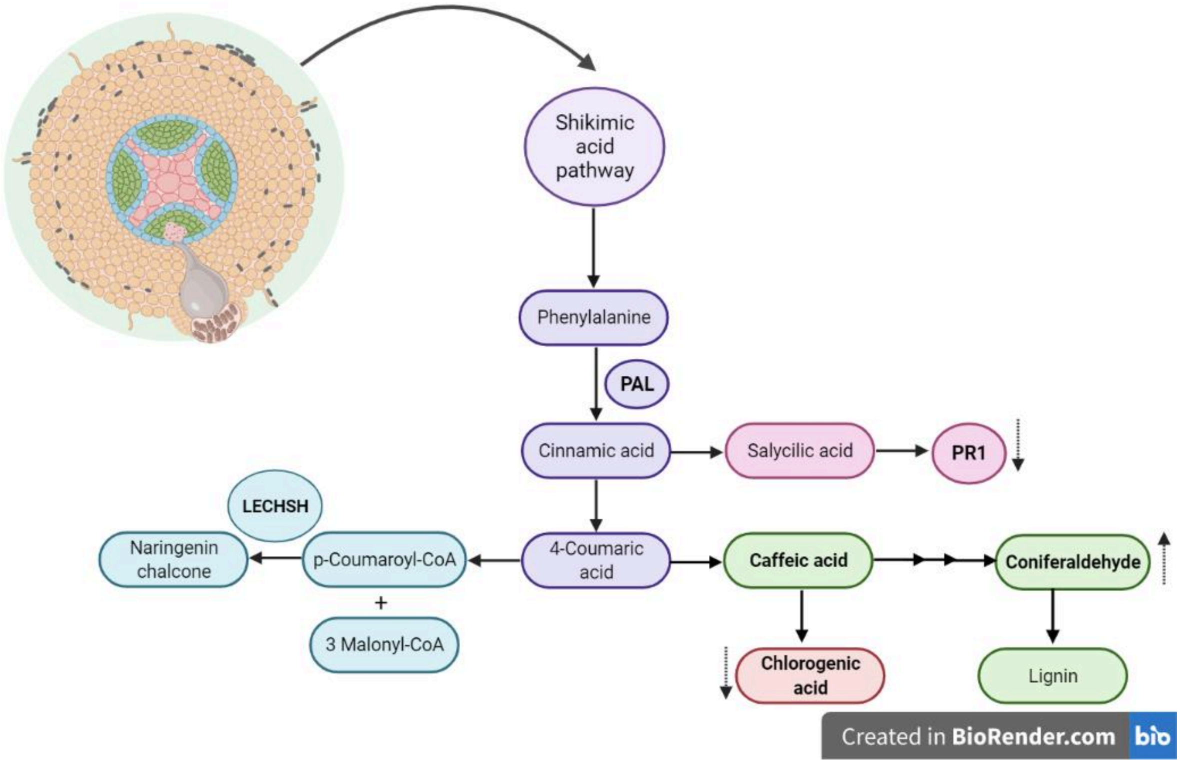

Figure 10: Summary of biochemical changes caused by *Pochonia chlamydosporia* colonization after 44 days in *Meloidogyne javanica* infected root. Compounds in bold correspond to the

compounds evaluated in the study. Up arrow indicates compound increase due to fungus presence and down arrow indicates compound decrease due to fungus presence.

In the second evaluation period, the nematode infected roots under fungal colonization showed lower expression of the ACC oxidase gene (last step for ethylene synthesis) and lower concentration of ACC (precursor of ethylene). Thus, there is a possibility of lower ethylene concentration in roots infected by *M. javanica* and colonized by *P. chlamydosporia* after 44 days of interaction. In tomato roots colonized by this fungus, lower expression was also detected for different genes that refer to ACC oxidase in the interval of 4, 7, and 21 days of interaction (Pentimone et al., 2019). For plant-parasitic nematodes, ethylene is related to the attractiveness of the nematode to the root. However, the attractiveness or repulsion depends on the nematode species and the genes deleted in the mutant plant (Gheysen et al., 2019). ERF-E2 knockdown increased the attraction of *M. incognita* and *Globodera pallida* to tomato root exudates (Dyer et al., 2019). Nevertheless, for *M. javanica* there was no change in attraction (Dyer et al., 2019). On the other hand, the ethylene insensitive mutant Never ripe (tomato) was more attractive to *M. javanica* (Cepulytė et al., 2018).

Thus, the consequences of this possible reduction of ethylene should be better investigated to confirm whether ethylene would have the same function related to attractiveness in plants already infected by the nematode.

Two developmental stages of nematode infection were evaluated, corresponding to galls in the initial phase of formation and at the mature when the egg masses are present at the surface of the galls. The fungus colonization resulted in fewer of these structures in both evaluation periods. In addition, with the non-target metabolomics analysis, it was possible to identify that the galls from plants inoculated with the fungus had different metabolite abundances in relation to the root galls from a plant not colonized by the fungus. From the metabolomics data, it was possible to identify some metabolites, such as the cis-5, 8, 11, 14-eicosatetraenoic acid (arachidonic acid) in which galls from plants treated with the fungus have a greater abundance of this compound. Tomato seeds incubated with this acid and then cultivated showed a 33% reduction in the number of galls of *M. incognita* (Vasyukova et al., 2009). Therefore, an increase of arachidonic acid may have helped to reduce the number of galls, as observed in the treatment with *P. chlamydosporia*.

Epicatechin was other identified compound that increased in galls in the presence of the fungus, in the first evaluation period. This compound reduces the hatching of *M. incognita* eggs, and in tomato roots infected with *M. incognita*, this compound is reduced (Masler et al., 2013;

Khanna et al., 2019). It is possible to infer a relationship between epicatechin and changes in nematode development. Thus, the increase of this compound in the galls may be a contribution of the fungus to contain the advance of the nematode development.

In galls from plants colonized by *P. chlamydosporia*, an increase in phytosphingosine was detected after 44 days of interaction. *Bacillus cereus* produces phytosphingosine and sphingosine and the fraction with these compounds caused mortality in *C. elegans* and *M. incognita* (Gao et al., 2016). Interestingly the soybean plants inoculated with *Bacillus simplex* also triggered the increase in the phytosphingosine level, which contributed to greater resistance to the cyst nematode (Kang et al., 2020). Phytosphingosine is a sphingolipid that serves as a signaling molecule linked to cell death processes and the regulation of defense reactions against pathogens (Sánchez-Rangel et al., 2015). Magnin-Robert et al. (2015) demonstrate that sphingolipids play an important role in plant defense involving the jasmonate signaling pathway and cell death. Thus, alteration in the JA levels in plants under fungal colonization may be related to the increase of the phytosphingosine levels inducing plant defense against nematode infection.

The highest abundance of 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine in root galls colonized by the fungus was detected in the first and second evaluation periods. This metabolite is a lysophosphatidylethanolamine formed from the action of phospholipase A2 and in tomato plants, it can delay the senescence of tomato leaves and fruits (Farag 1993). Exogenous application of this compound to radish increased phenylalanine ammonia lyase (PAL) activity, increased pathogenesis-related protein (PR) expression, and increased amounts of sinapine and lignin were detected (Hong et al., 2009). If this compound acts similarly in the galls, it can affect the development of the nematode. Another possible compound detected was 1-Hexadecanoyl-sn-glycero-3-phosphoethanolamine, belonging to the lysophosphatidylethanolamine family, whose function may be similar to 1-palmitoyl-2-hydroxy-sn-glycero-3-phosphoethanolamine due to the chemical similarity between the compounds.

In the second evaluation period in galls, there was an increase in the compound N-octadecanoyl-L-homoserine lactone in the presence of the fungus. It is a signaling molecule produced by gram-negative bacteria and secreted during the quorum sensing process responsible for sensing population density (Palmer et al., 2014). The presence of this molecule can be justified from metagenomic data of galls formed by *M. incognita*, which identified that the microbial composition of galls is different from that of roots, with bacteria belonging to the order *Enterobacteriales* being detected in greater abundance in galls and practically absent in non-galled regions of the roots (Tian et al., 2015). Previous studies suggest that *Enterobacteriales* were commonly associated with root-knot nematode cuticle as part of their lifestyle, so these bacteria enter the inner root tissue as bacterial associates of nematode infection and may help the pathogen establish its nursing cells (Adam et al., 2014; Cao et al., 2015; Tian et al., 2015).

There is a great diversity of these signalling molecules (acyl homoserine lactone) which, in addition to being important in the bacterial communication mechanism, are also capable of promoting plant growth and conferring resistance to the fungus *Alternaria alternata* in tomato plants (Palmer et al., 2014; Ortiz-Castro et al., 2008; Schuhegger et al., 2006). Soybean root invasion by *Pratylenchus penetrans* was reduced in plants pretreated with N-3-oxo-tetradecanoyl-L-homoserine lactone (oxo-C14-HSL) due to a faster and stronger defense induction of root invasion by nematodes (Adss et al., 2021).

In addition to the alterations caused by the fungus in the galls, we investigated whether the fungal colonization in the roots infected by the nematode causes systemic changes. For this, the metabolomic analysis of the leaves was performed. In this evaluation, we confirmed that the abundances of metabolites in leaves where the roots were colonized by the fungus and infected by nematode are different from where only nematode infection occurred in the two evaluation periods. In general, the profiles were different in presence of the fungus, as also observed by Gouveia et al. (2022). Among the altered metabolites in the leaves, the M416T40 ion possibly corresponds tomatidine, an intermediate in the synthesis of α-tomatine, main steroid glycoalkaloid present in tomato leaves. This compound has antifungal action and can be used as a defense mechanism by the plant. In the first evaluation period, the M578T40 ion also increased significantly in the leaves when there was fungal colonization in the root. Due to the similarity between fragments of the M416T40 ion and M578T40 ion, this ion may correspond to tomatidine galactoside. Therefore, the increased production of two α-tomatin biosynthesis intermediates may indicate greater activation of this pathway in leaves from plants whose roots were colonized by *P. chlamydosporia* and infected by the nematode.

The infection of *M. incognita* in the roots changed volatiles in the leaves of tomato, which resulted in lower oviposition of *Tuta absoluta* and a higher death rate of pupae (Arce et al., 2017). Furthermore, in the banana plant, it was demonstrated that the presence of *P. chlamydosporia* and *M. incognita* in the root contributed to the higher expression of the proteinase inhibitor II (PIN II) and pathogenesis-related proteins 1 (PR1) genes in the leaves

(Tolba et al., 2021). These results and the possible increase of tomatin in tomato leaf establish a perspective for new studies on increased defenses to foliar pathogens in this plant-fungus-nematode interaction.

5. Conclusion

The study of the interaction between *P. chlamydosporia* and *Solanum lycopersicum* infected by *M. javanica* identified activation of critical pathways for defense in plants. The fungus in nematode infected roots altered the expression of genes and the production of compounds (phenolic and flavonoids) that are important for the control of the root-knot nematode. In addition, the regulation of jasmonic acid in the presence of the fungus may be essential for the plant's defense responses. Metabolic alterations that occurred in galls due to colonization of the roots by the fungus indicate the action of indirect mechanisms of the fungus that can interfere with the development of nematode. Biochemical analysis showed that *P. chlamydosporia* altered different metabolic pathways in the roots. These modifications are extended to the leaves since the leaf metabolome was altered when the fungus colonized the root. This study showed that even with the suppression of some plant defense systems by the endophytic fungus, the interaction was still advantageous due to the many other benefits that the fungus provided to the infected plant, resulting in lower reproduction of *M. javanica.*

6. Supplementary material

Table S1. Transition list used as input in the Skyline to the analysis of the phytohormones.

Molecule Name	Precursor Name	Precursor m/z	Precursor Charge	Product m/z	Product charge	Precursor RT (min)	Exact mass
JA	Jasmonic Acid	209	-1	59	-1	9.2	210.12
ABA	Abscisic acid	263.1	-1	153	-1	8.2	264.13
SA	Salicylic acid	136.8	-1	92.9	-1	6	138.03
IAA	Indoleacetic Acid	176	1	129.9	1	7.5	175.06
ACC	ACC	102.1	1	56.2	1	0.6	101.04

Table S2: Transition list used as input in the Skyline to the analysis of the compounds.

Molecule List Name	Precursor m/z	Precursor Charge	Product m/z	Product charge	Precursor RT	Explicit collision energy
4 - hydroxybenzoic acid	139.12	1	121	1	3.85	20
4 - hydroxyflavone	239.07	1	137	1	9.89	20
4-hydroxy-3-methoxy-cinnamaldehyde	179.1	1	147.04	1	7.19	10
7 - hydroxyflavone	239.07	1	137	1	9.89	20
Benzoic acid	123.02	1	77	1	6.82	30
Caffeic acid	179	-1	135	-1	4.61	10
catecol	109.02	-1	91.01	-1	3.52	30
chlorogenic acid	355	1	163	1	3.86	20
coumarin	147.06	1	91	1	7.74	20
curcumin	367.11	-1	173.06	-1	12.46	10
Ferulic acid	193.05	-1	134.1	-1	6.31	20
Isoferulic	193	-1	134	-1	6.31	20
Neochlorogenic acid	353.1	-1	179	-1	3.05	10
N-propil galato	213	1	153	1	7.49	20
p-coumaric acid	163.04	-1	119	-1	5.81	10
sinapic acid	223	-1	164.1	-1	6.34	10
sinapyl alcohol	211	1	133	1	13.91	20
syringic acid	197	-1	121.2	-1	4.7	10
Trans-cinnamic acid	147	-1	77	-1	8.78	30
vanillin	151	-1	92	-1	5.59	20

3.5-dihydroxy benzoic acid	155.02	1	137.01	1	2.65	10
Hesperidin	611	1	303	1	6.8	30
Rutin	611	1	303	1	6.2	30
Naringin	581	1	273	1	6.8	30
Orientin	449	1	329	1	5.8	30
Isoorientin	449	1	299	1	5.6	30
Vitexin	433	1	313	1	6.2	30
Isovitexin	433	1	283	1	6.2	30
Myricetin	319	1	153	1	7.2	30
Morin	303	1	153	1	8	30
Hesperetin	303	1	153	1	6.8	30
Quercitin	303	1	153	1	8	30
Epicatechin	291	1	139	1	5	30
Catechin	291	1	139	1	4.2	30
Kaempferol	287	1	153	1	8.6	30
Luteolin	287	1	153	1	7.9	30
Phloretin	275	1	107	1	7.1	30
Narigenin	273	1	153	1	8.4	30
Genistein	271	1	153	1	8.4	30
Apigenin	271	1	153	1	8.4	30
Daidzein	255	1	137	1	7.7	30
Chalcone	210	1	104	1	11.2	30

Table S3. List of primer sequences used for qRT-PCR analysis.

Gene	Forward primer(5’-3’)	Reverse primer(5’-3’)
Actin	CGGTGACCACTTTCCGATCT	TCCTCACCGTCAGCCATTTT
RBOH1	GGAGCTCCAGCACAAGATTA	CTTGTTGCAGCACTCATGTC
PI1	GAAACTCTCATGGCACGAA	CCTTCGCACATCAAGTTAGAG
PR1	TCCGAGAGGCCAAGCTATAA	GACTGAGTTGCGCCAGACTA
PAL	TGAAGAGTGTTGTCAAGAACACAGT	AGCACCATTCCAGCTCTTCAGACA
LeCHS-2	TTCGGTTAAGCGGCTCATGA	CTCGAGCACCCTTGTTGTTCTC
ACC-oxidase	ACGGGACTCGGATGTCATTA-	TGCAATTGGATCACTTTCCAT

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CAPÍTULO 3

RESUMO

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, junho de 2022. **Genótipo de soja infectado pelo nematoide das lesões radiculares, *Pratylenchus brachyurus*, desencadeia uma cascata de defesa vegetal dependente de ácido salicílico e aumenta a produção de flavonoides e fitoalexinas.** Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes e Maria Goreti de Almeida Oliveira.

Pratylenchus brachyurus é uma importante espécie de nematoide que causa perdas significativas na produtividade da soja, principalmente no Brasil. As alterações bioquímicas que ocorrem na interação entre raízes de soja e esse nematoide são pouco conhecidas. Neste estudo, foram comparadas as respostas bioquímicas de dois genótipos de soja (MG/BR 46 e BRS 7380), com diferentes taxas de reprodução para nematoide da lesão radicular, com o objetivo de identificar cascatas de defesa da planta que reduziram a patogenicidade e modificações metabólicas importantes para a desenvolvimento do nematoide. O nível de auxina aumentou durante a infecção do nematoide em ambos os genótipos, indicando que este fitohormônio é importante para o ciclo de vida do nematoide. A desregulação do metaboloma radicular e o menor fator de reprodução do nematoide correlacionaram-se com o maior nível de ácido salicílico na BRS 7380. De fato, os perfis de compostos fenólicos diferiram entre os genótipos, incluindo precursores para biossíntese de lignina e flavonoides. Os níveis de flavonoides foram maiores no genótipo BRS 7380, e houve redução na reprodução de *P. brachyurus* em raízes pré-tratadas com as agliconas. Além disso, os flavonoides são precursores da síntese de fitoalexinas que presentaram maiores concentrações no genótipo BRS 7380 sob infecção por *P. brachyurus*. Assim, essas vias desreguladas podem estar envolvidas em mecanismos moleculares que contribuem para a menor reprodução do nematoide nesta cultivar.

Palavras-chave: *Pratylenchus brachyurus*. Flavonoides. Fitolalexinas. Daidzeína

ABSTRACT

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, June, 2022. **Soybean genotype infected by the root lesion nematode, *Pratylenchus brachyurus*, trigger an salicylic acid-dependent plant defense cascade and increases the production of flavonoids and phytoalexins.** Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes and Maria Goreti de Almeida Oliveira.

Pratylenchus brachyurus is an important nematode species that cause significant reductions in the soybean productivity, mainly in Brazil. The biochemical changes in the interaction between soybean roots and this nematode species have been barely known. In this study, the response biochemical of the two soybean genotypes (MG/BR 46 and BRS 7380), with different reproduction rates to the root lesion nematode were compared to identify plant defense cascades that reduced the pathogenicity and metabolic modifications important for the development of the nematode. The auxin level increased during nematode infection in both genotypes, indicating that this phytohormone is important to the nematode life cycle. Dysregulation of the root metabolome and lower nematode reproduction factor correlated with the higher salicylic acid level in the BRS 7380. In fact, phenolic compound profiles differed between the genotypes, including precursors for lignin and flavonoid biosynthesis. Flavonoid levels were higher in the BRS 7380 genotype, and there was reduced reproduction of the *P. brachyurus* in roots pre-treated with the aglycones. Moreover, flavonoids are precursors for synthesizing phytoalexins and presented higher concentrations in BRS 7380 genotype under infection by *P. brachyurus*. Thus, these dysregulated pathways may be involved in molecular mechanisms that contribute to the lower reproduction of the nematode in this cultivar.

Keywords: *Pratylenchus brachyurus*. Flavonoids. Phytolalexins. Daidzein.

1. Introduction

Soybean is one of the most important crops for grain production in Brazil and the United States. However, the productivity may be dramatically reduced by the attack of nematodes (Nicol et al., 2011; Oliveira et al., 2021). The control of this pathogen is challenged and requires crop management, such as the use of resistant genotypes in regions containing the pathogen (Oliveira et al., 2021). Among these nematodes, the root lesion nematode, *P. brachyurus*, is one of the main responsible for the reductions in the productivity of soybean. This nematode is a migratory endoparasite that penetrates the root tissue and migrates intracellularly, feeding on the cytoplasm of root cortex cells (Fosu-Nyarko & Jones, 2016).

In this interaction, plants respond to the invasion of nematodes in a complex way, involving transcriptional reprogramming and activation of the different biochemical pathways that can contribute to the plant defense response. In alfalfa infected by *Pratylenchus penetrans*, genes related to the phenylpropanoid pathway were induced, and there was an accumulation of secondary metabolites in cells (Vieira et al., 2019). In moderately resistant wheat to *Pratylenchus thornei* there was a higher concentration of quercetin-3,4'-Odi-beta-glycoside, linoleic acid, lysophosphatidylethanolamine, hirsutin, and 1-methylsulfnylbutenyl-isothiocyanate in roots infected by the nematode. These changes may contribute to the strengthening of the root cell wall and consequently inhibit penetration and/or reduce nematode motility (Rahaman et al., 2021).

This parasitism affects root functionality, and symptoms similar to nutrient deficiencies and water stress are common in infected plants. In the addition, penetration of root lesion nematode causes root damage that predisposes it to secondary infections from soil fungi and bacteria (Fosu-Nyarko & Jones, 2016; Jones & Fosu-Nyarko, 2014). The control of the *Pratylenchus* with nematicides (chemical and biological) and the use of resistant cultivars can reduce the population. However, genes related to resistance to *Pratylenchus* spp. have not been identified so far in soybean, and there is no record of resistant cultivars on the market, which offers only a few cultivars that have low reproduction factors for the nematode (Dias-Arieira et al., 2018; Machado et al., 2016).

In this study, soybean cultivars BRS 7380, which was registered as having low reproduction factor of *Pratylenchu*s and MG/BR 46, considered more susceptible to this nematode, were used to compare the defense responses against *P. brachyurus* infection. Changes in hormonal profile, phenolic compounds, and flavonoids were investigated during the infection of soybean roots. Overall, the biochemical and metabolic analyzes indicated plant defense cascades, acting in soybean plants to reduce the nematode reproduction.

2. Methodology

2.1 Nematodes and plants

The nematode *P.brachyurus* was multiplied in soybean plant in greenhouse. After extracting the nematodes the suspension was calibrated with the aid of an optical microscope to obtain a final suspension containing 500 nematodes per mL. In this study, the soybean genotypes MG/BR 46 and BRS 7380 were used.

2.2 Greenhouse experiments

For the greenhouse experiments, the following treatments were used: MG/BR 46; MG/BR 46 + *P. brachyurus*; BRS 7380 and BRS 7380 + *P. brachyurus*. 2L plastic pots containing sterilized substrate composed of C horizon clayey soil and washed sand in a ratio of 1:1 (V: V) were used, and one seed was added per pot. After 12 days, 500 specimens of *P. brachyurus* were inoculated per soybean seedling (V1 stage), obtained from seeds previously disinfected with gaseous chlorine. The experiments to evaluate the reproduction of *P. brachyurus* were conducted in two periods, the first from November 2020 to January 2021 (Experiment 1) with 56 days and the second from October 2021 to January 2022 (Experiment 2) with 80 days. The reproduction factor (RF) was calculated by the ratio between the *P. brachyurus* final (FP) and initial populations (IP) (RF = FP/IP).

The experiment 1 was evaluated at three different times, 7, 21, and 56 days after nematode inoculation, and in these respective periods, the entire roots were collected and immediately frozen with liquid nitrogen for biochemical analyses. Within 56 days, specimens of *P. brachyurus* were extracted by the method proposed by Coolen and D'Herde (1972) and quantified in a Peters chamber with an optical microscope. The experiment 2, reproduction factor, dry shoot mass, fresh root mass, and the number of pods per plant were evaluated. The experimental design was randomized and three replications were used for biochemical analysis, each repetition formed by a pool of three plants. For the evaluation of nematode reproduction, seven replicates were used.

2.3 Biochemical analyzes of the root

2.3.1 Total phenolics compounds

The total phenolic compounds were extracted from 100 mg of the root of each treatment (MG/BR 46; MG/BR 46 + *P.brachyurus*; BRS 7380 and BRS 7380 + *P.brachyurus*) using 80% methanol (v/v). The tubes were shaken for 12 h. After centrifugation, the supernatant was recovered and used to analyze total phenolics by the Folin-Ciocalteu method (Makkar et al., 1993). Afterward, the absorbance was measured using a UV Spectrophotometer (Shimazu, UV-1800) at 760 nm against a blank without extract.The standard curve of gallic acid (mg/L) was used for quantification, and the result was expressed in mg of gallic acid equivalents per gram of fresh root (mg GAE/g).

2.3.2 Polyphenoloxidase (PPO) Activity

For PPO activity, 100mg of the root was homogenized with 0.1 M potassium phosphate buffer (pH 7). The supernatant obtained after centrifugation at 14,000 g for 15 min at 4 ◦C was used for the enzymatic reaction. The reaction was composed of 0.1 M potassium phosphate (pH 7.0) and 120 mM pyrocatechol. The reaction was initiated by adding enzyme extract, and the activity was measured in a spectrophotometer at 420 nm for 2.7 minutes. PPO activity was expressed as U/mL, and U was defined as changes in absorbance at 420 nm min^{-1} (Benjamin & Montgomery, 1973).

2.3.3 Lignin

The pellet obtained from the methanol extraction (2.3.1) was used to determine the lignin content by the thioglycolic acid method (Campbell et al., 1992). Lignin was quantified as absorbance values (280nm) per gram of dry root (A280nm g^{-1}).

2.3.4 Evaluation of compounds by LC/MS

Flavonoids, phenolic compounds, phytoalexins and hormones were extracted from 200 mg of root with 700 µL of extractor solution (methanol, isopropanol, and acetic acid solution (20: 79: 1), according to Vital (2019). The metabolites were separated by ultra-performance chromatography (UHPLC-Agilent), using a C18 column (50 mm x 1.0 mm ID, 1.7 µm particle, and 300 A), coupled online to a triple quadrupole mass spectrometer (QqQ). The equipment was operated in MRM mode (multiple reactions monitoring). The spectra referring to the phenolic compounds, phytoalexins and hormones were analyzed in the Skyline software, which allowed the quantitative analysis of the compounds in the root. The flavonoid profile was performed according to the methodology of Gómez et al., 2018. Each class of compound (flavonoid, phenolics, phytoalexins and hormones) was analyzed individually in the mass spectrometer.

2.4 Greenhouse experiment with pure compounds

The pure compounds, genistein, daidzein, coumestrol, and naringenin, were prepared at concentrations of 25 μg mL^{-1} and 50 μg mL^{-1} in 0.03% (V/V) dimethylsulfoxide (DMSO) solution. The control treatment was carried out with 0.03% DMSO solution. Soybean seedlings (Cultivar MG/BR 46) roots were submerged for 5 days in the solutions separately. Then they were transplanted into sterilized soil, and after 3 days of adaptation, 500 specimens of *P. brachyurus* were added per pot. After 50 days, the roots were collected, and specimens of *P. brachyurus* were extracted by the method proposed by Coolen and D'Herde (1972). The quantification of the specimens was made with the use of an optical microscope. Each treatment contained seven biological replicates.

P. brachyurus specimens were placed separately in contact with the solutions of genistein, daidzein, coumestrol, and naringenin at a concentration of 50 μg mL^{-1} and 0.03% DMSO at room temperature for 24 h. Soybean seedlings (Cultivar MG/BR 46) were inoculated with 500 nematodes pre-treated with these compounds, and the nematodes pre-treated with DMSO 0.03% constituted the control treatment. After 50 days, the roots were collected, and specimens of *P. brachyurus* were extracted by the method proposed by Coolen and D'Herde (1972). The quantification of the specimens was performed with the use of an optical microscope. Each treatment contained seven biological replicates.

2.5 Statistical Analysis

The means and standard deviations of the results were calculated, and the statistical analysis between the two means was performed using the T-Student test, with a significance of 5%, using the R version 3.6.0 program. When indicated, the data were submitted to analysis of variance (ANOVA) and Duncan's test with a significance of 5%, using the R version 3.6.0 program.

3. Results

3.1 Greenhouse experiment with *Pratylenchus brachyurus* in soybean genotypes

Soybean cultivars MG/BR 46 and BRS 7380 were selected to evaluate the differential multiplication of *P. brachyurus,* and to identify biochemical differences involved in plant defense responses. In the experiment 1, the total population of *P. brachyurus* infecting the roots was lower in the cultivar BRS 7380 when compared to the cultivar MG/BR 46 after 56 days (**Figure 1**). The same behavior of both cultivars observed in the experiment 1 was also found when this interaction was evaluated over a more extended period of time (80 days) (**Figure 1**). Thus, we confirm that under the conditions evaluated the cultivar BRS 7380 has a lower *P. brachyurus* reproduction factor compared to the cultivar MG/BR 46.

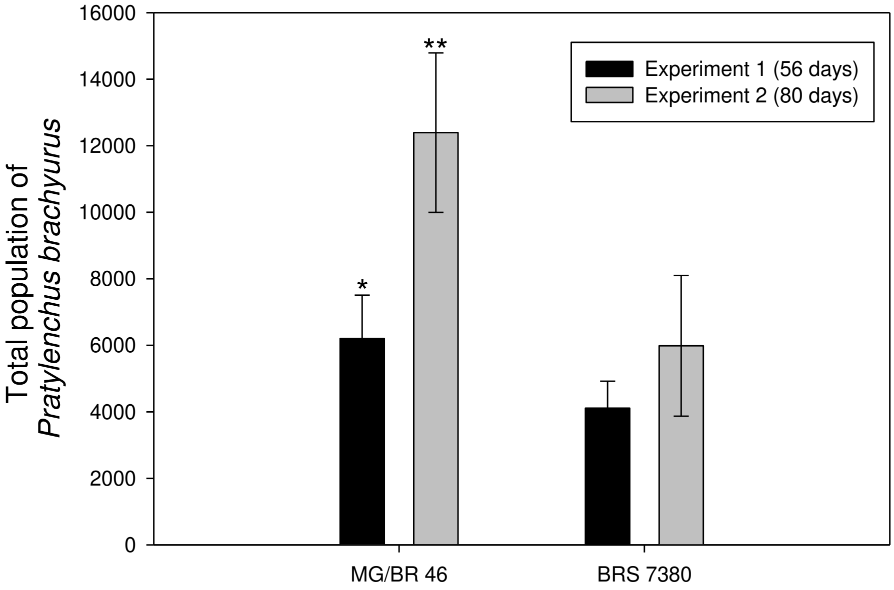

Figure 1: Total population of *Pratylenchus brachyurus* in soybean cultivars (MG/BR 46 e BRS 7380) in two evaluation periods (56 and 80 days). The average of the total number of specimens followed by only one asterisk differs from the Student's t-test ($p < 0.05$) after 56 days of nematode addition, and those followed by a double asterisk differ by the Student's t-test ($p < 0.05$) after 80 days of nematode addition. Bars represent the standard deviation (n =7).

In the experiment 2, when both cultivars were infected, there was no statistical difference in dry shoot mass. Root fresh mass of the cultivar BRS 7380 with *P. brachyurus* is lower than that of the infected cultivar MG/BR 46. When evaluating the number of pods per plant, it was identified that the infected BRS 7380 had more pods than the infected MG/BR 46 (**Table 1**).

Table 1: Dry shoot mass, soybean pod number, fresh root mass of cultivars MG/BR 46 and BRS 7380 after 80 days of infection by *Pratylenchus brachyurus.* According to the Student's t-test ($p < 0.05$) different letters indicate a significant difference between treatments ($p < 0.05$) at the respective evaluation.

	MG/BR 46	BRS 7380
Dry shoot mass (g)	2.747	2.482
Number of pods per plant	2.5^{b}	4.54 ^{a}
Fresh root mass (g)	13.706 ^{a}	6.785 ^{b}
Reproduction factor (56 days)	12	8
Reproduction factor (80 days)	25	12

3.2 Hormonal analysis in soybean roots

The experiment 1 was evaluated in three different periods to investigate the biochemical changes that occur in the plant throughout the nematode cycle. After 7 days of infection by *P. brachyurus*, the cultivars increased the production of salicylic acid (SA) and jasmonic acid (JA). At 21 days of analysis, BRS 7380 increased the production of SA and JA. However, there was no difference in the concentrations of the infected cultivar MG/BR 46. For the BRS 7380 genotype after 56 days, the levels for SA were markedly higher, indicating a plant defense cascade more activated, in accordance with the lowest reproduction of the nematode in this genotype (**Figures 2A**).

For the auxin hormone at 7 days, the infected cultivars showed opposite responses, since production of this hormone increased in BRS 7380, while for MG/BR 46 cultivar there was a reduction. At 21 and 56 days, the cultivars showed similar responses, an increase in the auxin production in the presence of the pathogen (**Figure 2C**). For the abscisic acid hormone, the cultivars infected by *P. brachyurus* showed the same pattern of response observed for auxin (**Figure 2D**).

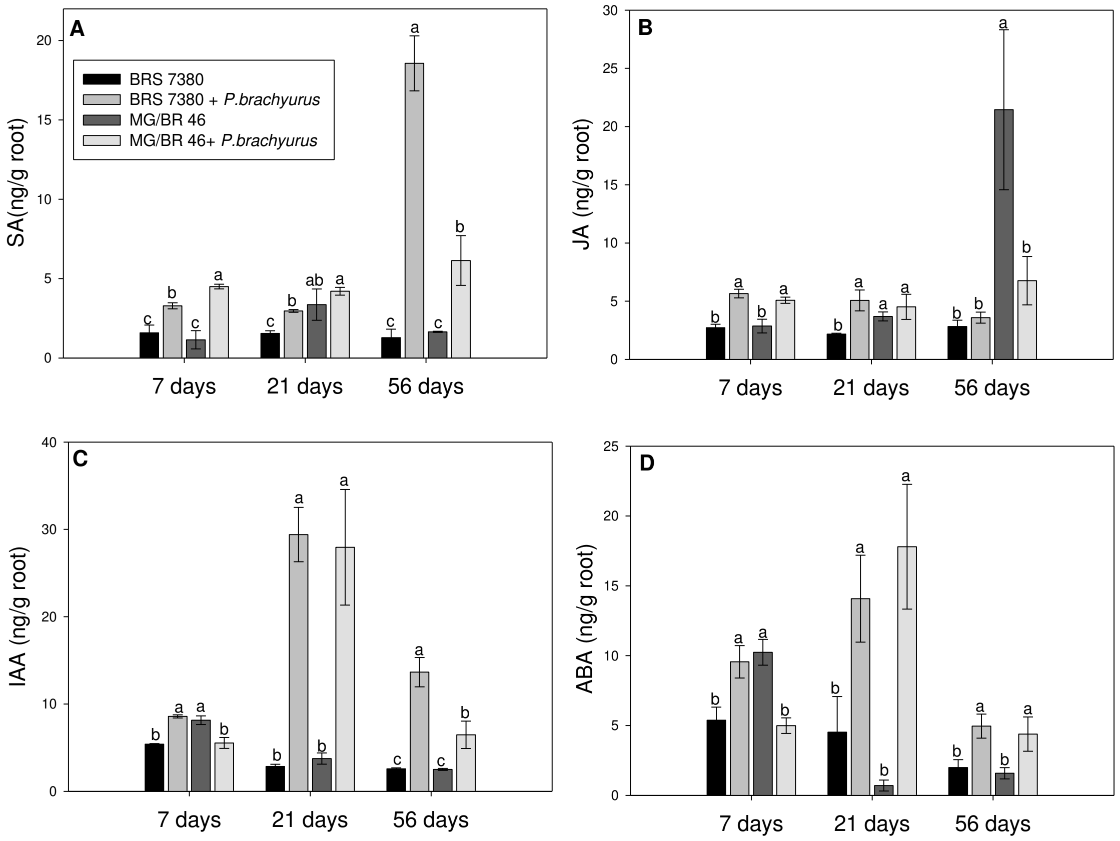

Figure 2: Quantification of phytohormones in soybean roots (MG/BR 46 e BRS 7380) without and with *Pratylenchus brachyurus* infection. **A)** Salicylic acid (SA); **B)** Jasmonic acid (JA); **C)** Indole-3-acetic acid (IAA) and **D)** Abscisic acid (ABA). According to the Duncan test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$) at the respective evaluation time. Bars represent the standard deviation (n =3).

3.3 Analysis of phenolic compounds in soybean roots

As expected, the nematode infection induced changes in the phytohormonal profiles. However, total phenolic levels were less responsive to nematode infection, being possible to observe higher levels for the BRS 7380 genotype (lower reproduction *P. brachyurus*) (**Figure 3A**). The concentration of total phenolics in BRS 7380 was higher than in MG/BR 46 in 21 and 56 days (**Figure 3A**). In the time evaluations of 7 and 21 days, the 3,5-dihydroxybenzoic acid was detected in higher concentration in the susceptible cultivar MG/BR 46 compared to BRS 7380, both infected by the pathogen (**Figure 3B**). On the other hand, cultivar BRS 7380 showed the best response to coumaric acid, as it increased the concentration of this compound in the presence of the nematode at 56 days concerning infected MG/BR 46 (**Figure 3C**). The compound 4-hydroxy-3-methoxy-cinnamaldehyde was detected only at 21 and 56 days. This compound increased in cultivar MG/BR 46 with *P. brachyurus* at 21 days, and at 56 days, it was reduced. For BRS 7380, there was a significant difference only in the last evaluation period due to pathogen infection (**Figure 3D**).

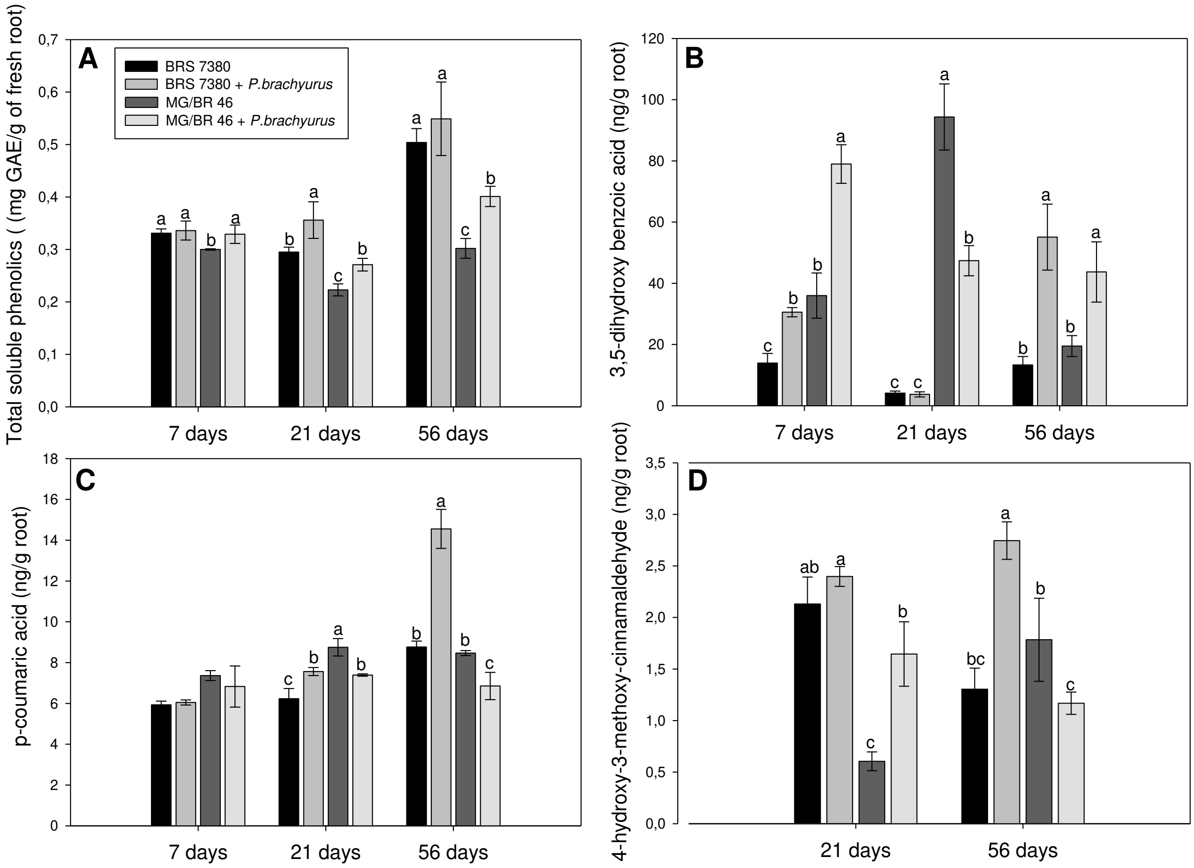

Figure 3: **A)** The concentration of total soluble phenolics in soybean roots (MG/BR 46 e BRS 7380) without and with *Pratylenchus brachyurus* infection and quantification of the phenolic compounds **B)** 3,5-dihydroxy benzoic acid; **C)** p-coumaric acid; **D)** 4-hydroxy-3-methoxy-cinnamaldehyde. According to the Duncan test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$) at the respective evaluation time. Bars represent the standard deviation (n =3).

3.4 Polyphenol oxidase quantification

In the first evaluation, both cultivars responded to the presence of the pathogen with increased polyphenol oxidase activity. However, in the following evaluations (21 and 56 days), there was a more significant activity of this enzyme in the cultivar BRS 7380 infected by *P. brachyurus* (**Figure 4**).

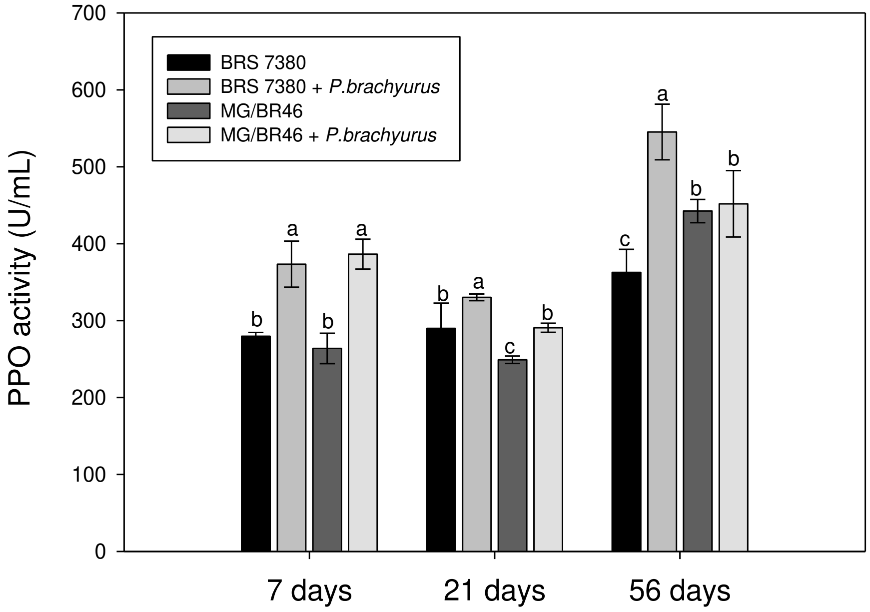

Figure 4: Polyphenol oxidase (PPO) activity in soybean roots (MG/BR 46 e BRS 7380) without and with *Pratylenchus brachyurus* infection. According to the Duncan test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$) at the respective evaluation time. Bars represent the standard deviation (n =3).

3.5 Quantification of lignin in soybean roots

As the infection process of the root lesion nematode involves intracellular migration through the root tissues, changes in the lignin content were evaluated in the roots of soybean cultivars since this compound provides greater mechanical resistance to the plant cell. In general, the nematode infection increased the lignin levels being higher at 21 days for BRS 7380, but at 56 days, it was equaly higher in both cultivars inoculated plants than in non-inoculated ones (**Figure 5**).

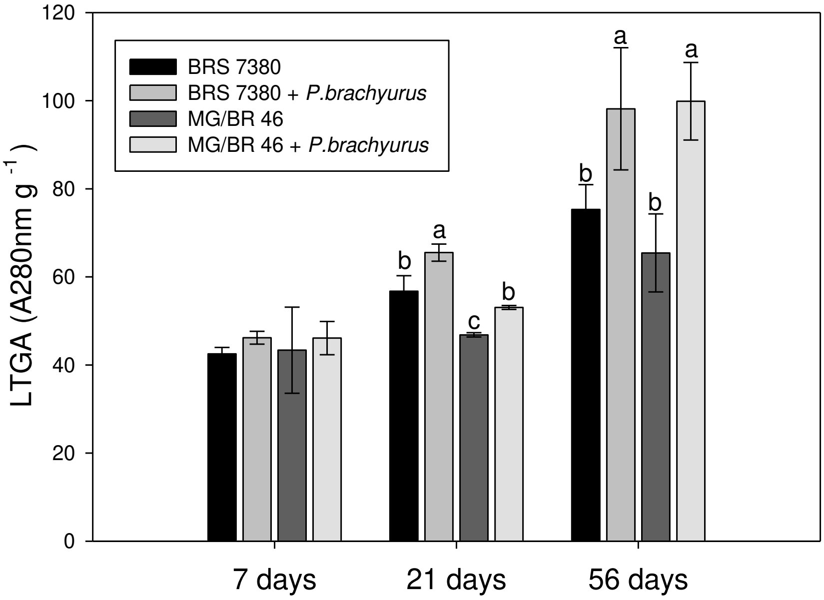

Figure 5: The lignin-thioglycolic acid (LTGA) derivative was quantified in soybean roots (MG/BR 46 e BRS 7380) without and with *Pratylenchus brachyurus* infection. According to the Duncan test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$) at the respective evaluation time. Bars represent the standard deviation (n =3).

3.6 Analysis of flavonoids in soybean roots

Initially, the root extracts were submitted to a metabolomic approach to evaluate some target flavonoid compounds, including the aglycones and some glycoconjugates (**Figure 6**). The methodology proposed by Gómez et al. (2018) was also applied to obtain more general profiles of the changed flavonoids during the nematode infection. The fragment relative intensities (FRI%) between the ions were evaluated manually to determine which m/z values are distinguished between the aglycone isomers (**Figure S1–S3**). From FRI%, the patterns of aglycone fragmentation of the glycoconjugates in the samples referring to 7 days of evaluation were identified, suggesting the presence of glycoconjugates containing the aglycones for Naringenin (RT=6; 7.24), Daidzein (RT=5.6; 6.3; 8.6; 9.4) and Genistein (RT= 6.6; 7) (**Figure S1**). For 21 days was identified Naringenin (RT=6; 7.24), Daidzein (RT=5.6; 6.4; 7.2), Genistein (RT=6.6; 7) and Luteolin (RT=5.8) (**Figure S2**). With 56 days Naringenin (RT=6; 7.24), Daidzein (RT=5.6; 6; 6.3; 6.5), Genistein (RT= 6.6; 6.9; 10.6) and Luteolin (RT=5.8) were detected in the samples (**Figure S3**).

The m/z value of each precursor ion, which the aglycone was previously characterized, was submitted to identifications against the Mass Bank repository, as indicated in **Table 2**.

Overall, the aglycones and glycoconjugates flavonoid profiles differed between the soybean genotypes.

Table 2: Identification of glycosylated flavonoids present in the roots of cultivars BRS 7380 and MG/BR 46 without and with *Pratylenchus brachyurus* infection in the three evaluation periods (7, 21, and 56 days).

Flavonoid Class	Precursor	RT (min)	Conjugate (m/z)	Mass	Relative Abundance in XIC (%)	Database
7 days						
Daidzein	255	5.6	417.2	416.11	100	Daidzein-7-O-glucoside
	255	6.3	503.2	502.11	70.99	Daidzein 7-O-glucoside-6''-O-malonate
	255	6.3	542.4		100	Not identified
	255	8.6	503	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
	255	9.4	503	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
Genistein	271	6.6	433.1	432.1	100	Genistein 7-O-glucoside
	271	7	519.3	518.1	100	Genistein 7-O-glucoside-6''-O-malonate
Naringenin	273	6	581.2	580.17	100	Naringin
	273	7.2	521		100	Not identified
21 days						
Daidzein	255	5.6	417.1	416.11	100	Daidzein-7-O-glucoside
	255	6.5	503	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
	255	7.2	517	516.12	100	Formononetin 7-O-glucoside-6''-O-malonate
	255	7.2	901.7		92.12	Not identified
Genistein	271	6.6	433.3	432.1	100	Genistein 7-O-glucoside

	271	7	519.4	518.1	55.01	Genistein 7-O-glucoside-6''-O-malonate
Luteolin	287	5.8	448.8	448.1	100	Luteolin-7-O-glucoside
Naringenin	273	6	581.2		100	Naringin
	273	6	521		33.48	
	273	7.2	521		100	Not identified
56 days						
Daidzein	255	5.6	417.2	416.11	100	Daidzein-7-O-glucoside
	255	6	503.2	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
	255	6	542.3		47.86	Not identified
	255	6.3	503.2	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
	255	6.3	542.2		32.29	Not identified
	255	6.5	503.3	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
Genistein	271	6.6	433.3	432.1	100	Genistein 7-O-glucoside
	271	7	519.4	518.1	100	Genistein 7-O-glucoside-6''-O-malonate
Luteolin	287	5.8	448.9	448.1	100	Luteolin-7-O-glucoside
Naringenin	273	6	581.2	580.1	100	Naringin
	273	7.2	521		100	Not identified

The compounds identified in the samples are constituents of flavonoid biosynthesis pathway that can provide precursors for synthesizing phytoalexins, which directly contribute to defense responses. Thus, coumestrol, glyceollin (I, II, III), and medicarpin were also investigated in the roots of cultivars infected by *P. brachyurus*. Together with the flavonoid analysis, these data were represented in the heatmap analysis (**Figure 6**). After the statistical analysis of the different compounds, the specific responses of the cultivars BRS 7380 and MG/BR 46 when infected by *P. brachyurus* were highlighted (**Figure 7**).

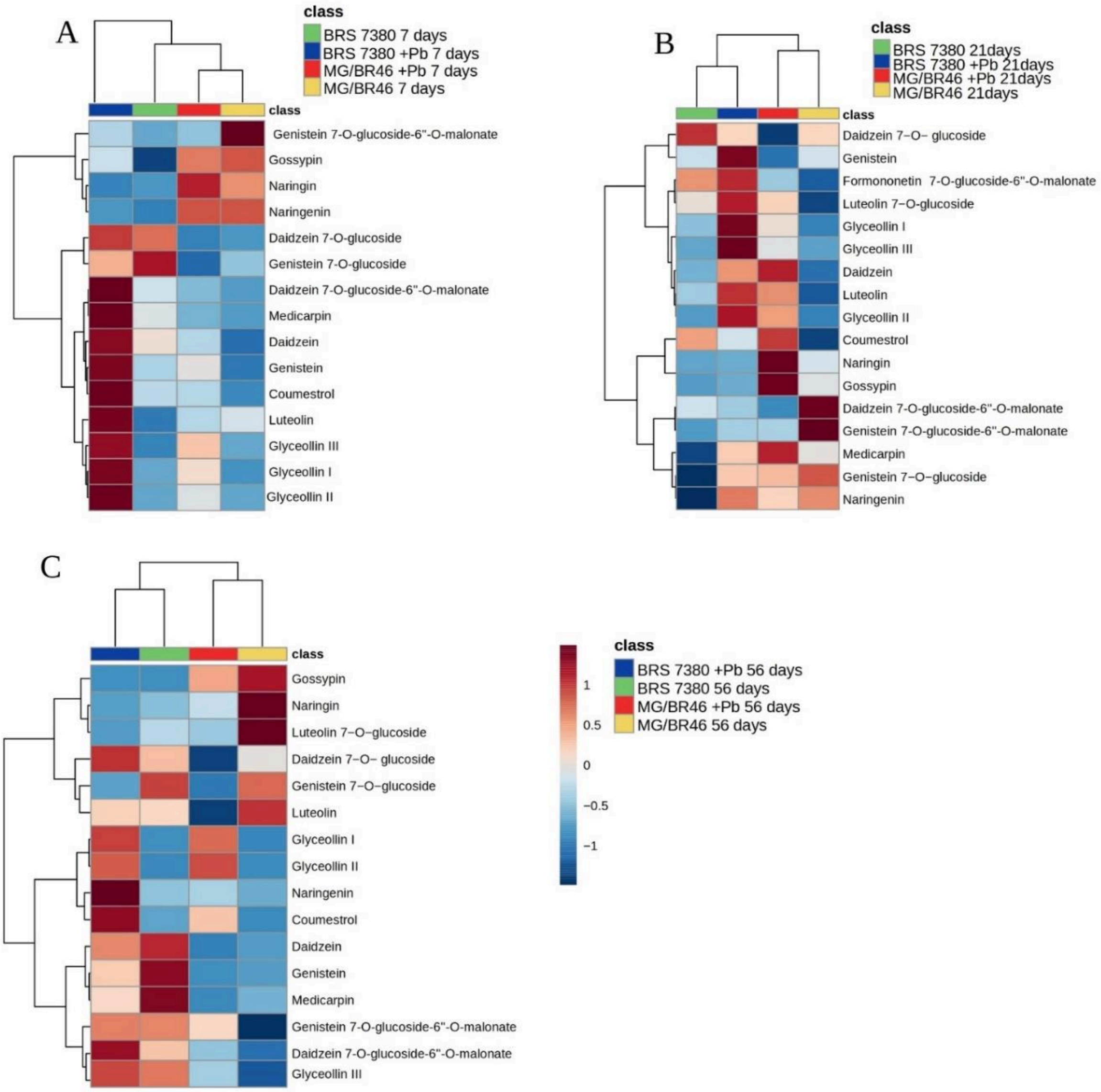

Figure 6: Cluster analysis by the HeatMap method of flavonoids characterized in soybean roots of genotypes MG/BR 46 and BRS 7380 in the presence or absence of *Pratylenchus brachyurus*. **(A)** 7 days, **(B)** 21 days, and **(C)** 56 days after nematode infection. The graph represents the differences in the abundance of metabolites analyzed by LC-MS in response to treatments, with blue color indicating reduction and red of increase.

In the first evaluation period (7 days), daidzein, a precursor of different biosynthesis pathways, showed higher concentrations in the infected BRS 7380. This precursor contributed to the higher concentration of glyceollin I, II, and III, daidzein-7-O-glucoside, daidzein 7-O-glucoside-6"-O-malonate, coumestrol, and medicarpin. In this cultivar, genistein, genistein 7-

O-glucoside, genistein 7-O-glucoside-6"-O-malonate and luteolin were also increased. Otherwise, in response to nematode infection, susceptible cultivar MG/BR 46 showed an increased concentration of naringenin and naringin in relation to cultivar BRS 7380 infected with the nematode (**Figure 7**).

During the second evaluation period (21 days), the infected BRS 7380 cultivar maintained the metabolic responses compared to the infected MG/BR 46 cultivar The concentrations of, glyceollin I, II, and III, daidzein-7-o-glucoside, and formononetin 7-o-glucoside-6 "-O-malonate were higher in the infected BRS 7380. Furthermore, the greater increase in naringenin in this cultivar may have contributed to the increase in luteolin and luteolin-7-O-glucoside. For daidzein, daidzein 7-O-glucoside-6"-O-malonate, coumestrol, genistein, genistein 7-O-glucoside, and genistein 7-O-glucoside-6"-O-malonate showed no statistical difference between the infected cultivars. The infected cultivar MG/BR 46 maintained a higher response to naringin, and there was an increase in medicarpin concentration compared to infected BRS 7380 (**Figure 7**).

Metabolic profiles of the soybean roots after 56 days for compounds glyceollin I and II, coumestrol, daidzein 7-O-glucoside-6-O-malonate, genistein 7-O-glucoside, genistein 7-O-glucoside-6"-O-malonate, and luteolin 7-O-glucoside there was no statistical difference between the infected cultivars. However, BRS 7380, which provided lower nematode reproduction, maintained more intense responses than MG/BR 46, such as higher concentration of daidzein, daidzein 7-O-glucoside glyceollin III, genistein, medicarpin, and luteolin. In MG/BR 46, the increase in naringenin and naringin predominated in response to the presence of *P.brachyurus* **(Figure 7**).

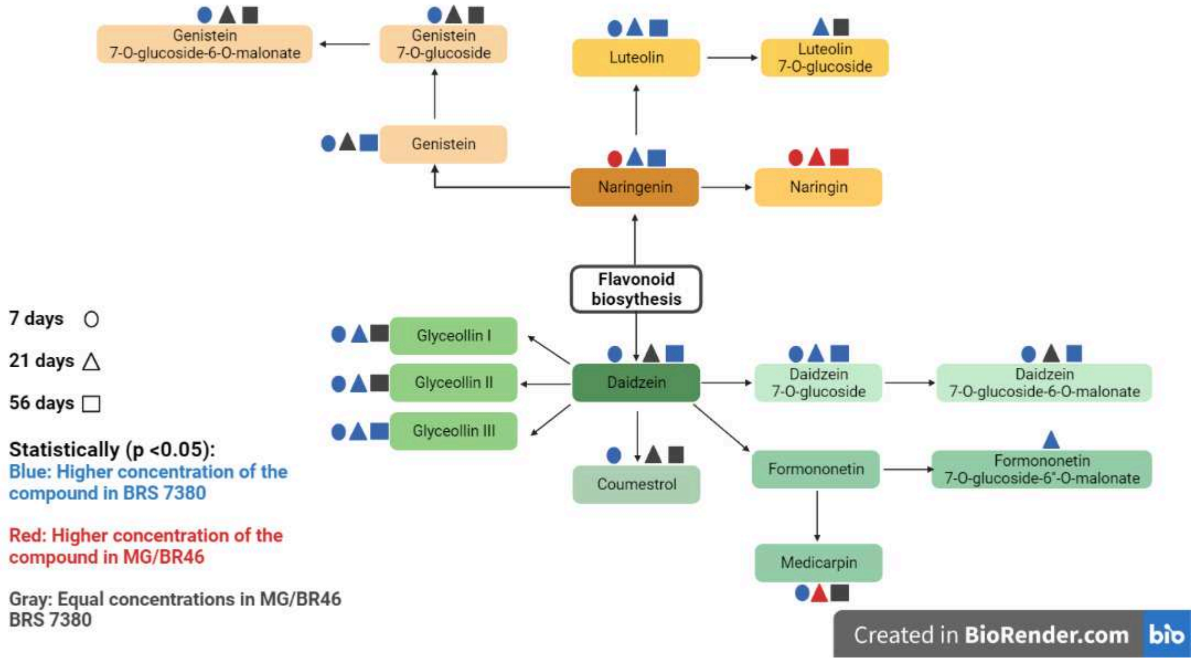

Figure 7: Compounds related to flavonoid biosynthesis were identified in extracts from soybean roots of the cultivar BRS 7380 and MG/BR 46 infected by *Pratylenchus brachyurus*. According to Duncan's test after ANOVA, the blue color represents the highest concentration for the cultivar BRS 7380 infected by *P. brachyurus*. The red color refers to the highest concentration in the cultivar MG/BR 46 infected with *P. brachyurus*. The gray color represents the absence of statistical difference between these two treatments. Circle refers to the 7 days evaluation period, triangle to 21 days, and square to 56 days.

3.7 Greenhouse experiment with pure compounds

As flavonoid levels were responsive to nematode infection, the effect of some compounds was evaluated in soybean roots on the nematode infection. Plants treated with the compounds (naringenin, daidzein, coumestrol, and genistein) at a concentration of 25 µg mL^{-1} reduced the nematode population compared to the control (DMSO 0.03%) (**Figure 8A**). Treatment with 25 µg mL^{-1} genistein more effectively reduced the *P. brachyurus* population. The experiment conducted with compounds (naringenin, daidzein, coumestrol, and genistein) in higher concentrations, 50 µg mL^{-1}, also reduced the population of *P. brachyurus*. However, there was no statistical difference between the pure flavonoids (**Figure 8B**).

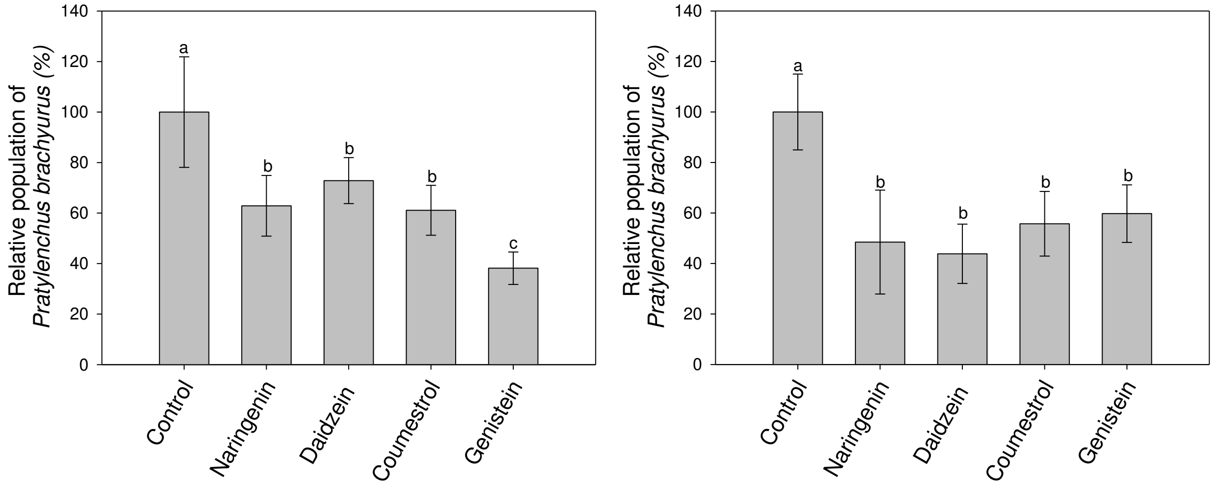

Figure 8: Total population of *P. brachyurus* in soybean roots (MG/BR 46) previously submerged for 5 days with synthetic flavonoids (naringenin; daidzein; coumestrol; genistein) at two concentrations: **A)** 25 µg $mL^{-1}$and **B)** 50 µg mL^{-1} and DMSO (0.03%). According to the Duncan test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$). Bars represent the standard deviation (n =7).

Thus, the reproduction of the nematode in plants with a higher concentration of flavonoids, naringenin, daidzein, coumestrol, and genistein may be harmed. Then, the nematicidal action of these compounds was investigated *in vitro* tests which specimens of *P. brachyurus* were kept for 24 h in solutions with these flavonoids at a concentration of 50 µg mL^{-1} and then inoculated into plants. The treatment of the specimens with the flavonoids did not harm the specimens since the final population of nematodes did not statistically differ from the control, in which the specimens were treated with 0.03% DMSO only (**Figure 9**).

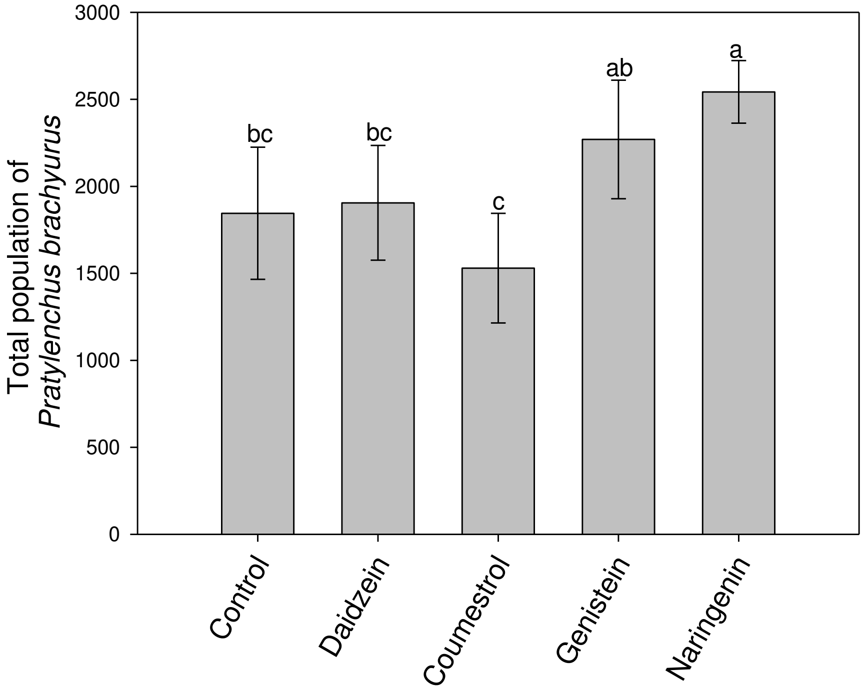

Figure 9: Total population of *P. brachyurus* in soybean roots (MG/BR 46) whose specimens were pre-treated for 24 h with genistein, daidzein, coumestrol, naringenin at 50 µg mL^{-1} and DMSO (0.03%). According to the Duncan test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$). Bars represent the standard deviation (n =7).

4. Discussion

Nematode infections may produce severe damage to soybean roots, reducing the productivity (Elhady et al., 2018). Therefore, understanding the biochemical changes in plants infected by *P. brachyurus* may contribute to developing resistant genotypes essential to maintaining productivity in an area with a high population density. Possible defense mechanisms of the two cultivars against *P. brachyurus* in the soybean roots were investigated. For this, cultivar BRS 7380 was used, which has a low reproduction factor for the root lesion nematode (*Pratylenchus* spp.) and the cultivar MG/BR 46 with a high reproduction factor for this nematode (Silva et al., 2015; Hamawaki et al., 2019).

Hormonal pathways are essential for producing plant defense responses against pathogen attacks, such as nematodes. Thus, comparing the hormonal profiles can provide information about the cascades of defense responses in cultivars infected by *P.brachyurus*. The infected cultivars, BRS 7380 and MG/BR 46, increased the production of jasmonic acid, but there was no statistical difference between the cultivars. The increase in the synthesis of this hormone in the initial periods of evaluation (7 and 21 days) may have contributed to the signaling of defense responses to contain the pathogen since this hormone is responsible for inducing systemic acquired resistance in plants (Chen et al., 2006). In wheat infected by *Pratylenchus thornei*, there was possibly an increase in jasmonic acid production, as higher production of alpha-linolenic acid (precursor of jasmonic acid) was detected in infected plants (Rahaman et al., 2020).

In general, in our evaluations over time, *P. brachyurus* infection caused an increase in auxin in both soybean cultivars, and changes related to this hormone were identified in other studies (Channale et al., 2021; Vieira et al., 2019). Transcriptomes from three chickpea cultivars showed upregulation of genes related to Small Auxin Upregulated RNA (SAUR) and other auxin-responsive genes when infected by *P. thornei* (Channale et al., 2021). In the roots of the alfalfa cultivar resistant to *Pratylenchus penetrans*, there was up-regulation of the flavin-monooxygenase gene, which is important in auxin biosynthesis (Vieira et al., 2019). A metabolomic evaluation of the roots of two wheat cultivars found that the cultivar susceptible to *P. thornei* has a higher amount of auxin in the roots compared to the moderately resistant cultivar (Rahaman et al., 2020).

From these data, we can suggest that auxin is related to the infection response of different species of *Pratylenchus*, and this hormone is fundamental in the cell expansion mechanism. In this process, auxin induces the SMALL AUXIN UP RNA (SAUR) proteins that inhibit the type 2c D-clade protein phosphatase (PP2C.D) activity, thus maintaining the plasma membrane H+-ATPases as a phosphorylated isoform and activated. This activates the H+ pump, which reduces apoplastic pH. In turn, pH reduction activates expansins and cell wall modifying enzymes that allow cell expansion (Spartz et al., 2014; McQueen-Mason et al., 1992; Cosgrove, 2016; Emenecker et al., 2020). If the auxin induced by the nematode infection in soybean contributes to the activation of expansins and the cell expansion mechanism. This may be a mechanism used by *P. brachyurus* to facilitate its movement and feeding in plant cells since its migratory lifestyle generates the need for the secretion of enzymes with actions on the cell wall throughout its development (Fosu-Nyarko & Jones, 2016).

For some species of the genus *Pratylenchus* there was the detection of expansins as effectors of this nematode. Possibly, during infection, these proteins help in the invasion of the nematode and the subsequent migration in the root tissue due to cell wall loosening activities. Expansins break the non-covalent bonds of fibrils and facilitate the access of other enzymes with cell wall activity (Vieira et al., 2018; Cosgrove, 2000). Vieira (2020) identified that the Pp-EXPB1 gene of *P.penetrans* encoded an expansin that possibly acts on the movement and feeding of the nematode by extending and loosening the cell wall, in addition to acting as a virulence factor interfering with the host's immune response or immunity triggered by effectors contributing to the advancement of nematode development. In our evaluation, the trend of auxin and ABA results were the same at the three evaluation times.

The ABA hormone may also be involved in the mechanism described above, as this hormone can dephosphorylate and consequently inhibit plasma membrane H +-ATPases, which will result in fewer expansins activation (Hayashi et al., 2014; Emenecker et al., 2020). Based on our hormonal results, the two cultivars infected by the nematode showed higher production of ABA and auxin. The nematode may induce a more outstanding auxin production for activating plant expansins. On the other hand, the plant infected by the nematode increased the production of ABA to prevent excessive loosening of the cell wall.

In general, the hormonal responses between cultivars when infected by the nematode were similar. However, the SA level was higher in BRS 7380 infected at 56 days. Genes involved in SA biosynthesis and signaling have been important for the resistance of plants to infection by cyst nematode (Wubben et al., 2008; Kammerhofer et al., 2015). For the root-knot nematode, repression of SA signaling in tomato contributed to the successful development of the nematode and the addition of SA to the soil inhibited the reproduction of this nematode (Molinari et al., 2014; Molinari, 2016). Thus, it is possible that the increase of salicylic acid in BRS 7380 at 56 days contributed to the lower reproduction of the nematode in this cultivar.

Phenols are secondary metabolites widely spread in plants and they are important in defense of plants against pathogens, including root lesion nematodes (*P. penetrans, P. coffeae, P. zeae* and *P.thornei*) (Acedo et al., 1971; Backiyarani et al., 2013; Vaganan et al., 2014; Mehta et al., 2005; Rahaman et al., 2020).

In cultivar BRS 7380, which had a lower nematode reproduction, there is a higher concentration of total phenolics concerning infected MG/BR 46 (21 and 56 days). Furthermore, it was observed that the highest concentration of these compounds was characterized as a constitutive response in BRS 7380, and this was observed in *P. thornei* resistant wheat genotypes (Rahaman et al., 2020). In the cultivar with the lowest reproduction factor (BRS 7380), the defense mechanisms may be associated with a higher concentration of phenolic compounds. Furthermore, polyphenol oxidases can oxidize these compounds and form quinones with antimicrobial activity (Constabel & Barbehenn et al., 2008).

The increase in PPO activity is often reported as a plant defense response to the invasion of pathogen, and the BRS 7380 cultivar infected with the nematode had the highest polyphenol oxidase activity at 21 and 56 days. *P. thornei* resistant wheat also increased the activity of this enzyme in the presence of the nematode (Rahaman et al., 2020). Infection by *P. coffeae* in resistant bananas induced a more significant accumulation of polyphenol oxidase in relation to susceptible cultivars (Kumar et al., 2008). Thus, the increase in the activity of this enzyme can contribute to the formation of quinones that can be toxic to the nematode, such as naphthoquinone, which has a nematicidal activity for *P. thornei* (Esteves et al., 2017).

Among the deregulated phenolic compounds, coumaric acid and 4-hydroxy-3-methoxy-cinnamaldehyde were highly responsive to nematode infection. These compounds are coumarin and coniferyl alcohol precursors, respectively, monolignols that constitute lignin (Whetten et al., 1995; Sakamoto et al., 2020). At 21 days after nematode infection, the concentration of coumaric acid increased in the BRS 7380 genotype. However, it did not differ from that of the infected MG/BR 46, and the concentration of 4-hydroxy-3-methoxy-cinnamaldehyde was higher in the BRS 7380 cultivar, which produced this compound in a higher concentration constitutive. The increase in compounds used in the synthesis of lignin in the cultivar (BRS 7380) of lower reproduction factor to *P. brachyurus* may explain the higher deposition of lignin in this cultivar at 21 days in the presence of the nematode than cultivar MG/BR 46. This biochemical modification may have hampered the migration and development of the nematode, contributing to reducing the final population, as occurred in banana plants resistant to *P. coffeae* (Vaganan et al., 2014).

In the last evaluation period in the BRS 7380, the more significant deposition of lignin induced by nematode may have occurred due to the increased concentration of coumaric acid and 4-hydroxy-3-methoxy-cinnamaldehyde in this cultivar. Bananas resistant *to P. coffeae* have a higher amount of coumaric acid in the roots when compared to a susceptible cultivar (Vaganan et al., 2014). In addition to these compounds being used in lignin biosynthesis, they can be recruited for defense purposes, such as for the synthesis of other phenolic compounds, including phytoalexins, stilbenes, coumarins, and flavonoids (Weiergang et al., 1996; Dicko et al., 2005; Lozovaya et al., 2005; Lozovaya et al. al., 2007). At 56 days, the susceptible cultivar also presented mechanisms that resulted in greater lignin deposition, despite reducing coumaric acid and 4-hydroxy-3-methoxy-cinnamaldehyde when this cultivar was infected.

Flavonoids are a complex class of phenolic compounds showing diverse biological activities, including plant defense against pathogens. Thus, alterations of the flavonoid levels in the roots can be important to identify defense mechanisms in soybean when infected by *P. brachyurus*, which can be detected as free aglycones and as glycosidic conjugates. In addition, other modified forms (hydroxylated, methylated, prenylated) may occur. Glycosylation contributes to greater stability and solubility (water) of compounds, which is essential for preventing cytoplasmic damage and safe storage in the cell vacuole (lwashina, 2000).

In soybeans, the aglycones daidzein, genistein, and glycitein and their glycosides and malonate derivatives are the main flavonoids (Kudou et al., 1991; Suzuki et al., 2007; Yu et al., 2008). The cultivar BRS 7380 has a higher amount of daidzein in its roots than the cultivar MG/BR 46, which may have contributed to greater protection of BRS 7380 when infected by *P. brachyurus*. During nematode development, BRS 7380 maintained a higher concentration of daidzein at 7 and 56 days.

No nematicidal effect was observed when treating *P. brachyurus* specimens with 50 µg mL^{-1} of daidzein for 24 h because when inoculating the treated nematodes in soybean, there was no difference in the final population compared to the control (specimens treated with DMSO 0.03%). However, in the test in which plants absorbed daidzein (50 µg mL^{-1}) there was a 56% reduction in the total nematode population. These experiments may indicate that daidzein (50 µg mL^{-1}) under *in vitro* conditions has no nematicidal action against *P.brachyurus* but is essential for the plant's defense against the nematode.

Daidzein was also detected in the glycosylated form (daidzein 7-O-glucoside) in higher concentration in BRS 7380 when infected by the nematode throughout the evaluation. At 7 days of interaction, this cultivar had a higher concentration of daidzein 7-O-glucoside-6''-O-malonate. These chemical modifications contribute to the stabilization, solubility, and transport of the compounds. These compounds possibly play important roles in plant growth and adaptations to biotic and abiotic stresses, functions that are still being investigated (Ahmad et al., 2017).

Daidzein is an essential compound in synthesizing phytoalexins, such as coumestrol. This compound reduces the motility of *P. scribneri* and significantly increases in the roots of lima bean at the beginning of infection by this nematode (Rich et al., 1977). Increased coumestrol synthesis also occurred in *P. penetrans* infected soybean after treatment with N-3-oxo-tetradecanoyl-Lhomoserin lactone (Adss et al., 2021). In our evaluation, this compound did not show nematicidal activity when treating *P. brachyurus* specimens with 50 µg mL^{-1} of coumestrol for 24 h since these specimens generated a nematode population statistically equal to the control. However, there was a population reduction of 44% in plants that absorbed this toxin, which indicates that the nematode feeding on plant cells with a higher concentration of coumestrol can harm its development, unlike the *in vitro* test where the compound had contact only with the outside of the nematode, and there was no stimulus for the nematode to feed

At 7 days, *P. brachyurus* infection resulted in a higher concentration of coumestrol in BRS 7380 than in MG/BR 46, which may have contributed to the total reduction in the nematode population observed in this cultivar. In addition, at 56 days, both cultivars increased the synthesis of coumestrol. Possibly this phytoalexin may be acting as a defense response against *P. brachyurus*.

Infection by *P. brachyurus* in soybean cultivars increased medicarpin synthesis, possibly a defense response to the pathogen attack (Cook et al., 1995). *In vitro* tests verified the motility inhibitory action of *P. penetrans* by this phytoalexin, and a possible association between medicarpin levels and resistance to *P. penetrans* was observed in *Medicago sativa* (Baldridge et al., 1998). The cultivar MG/BR 46 showed in 21 days a higher concentration of medicarpin when infected, which can help in the defense responses of this cultivar.

Daidzein is the precursor of the phytoalexin glyceollin, which reduces motility and inhibits oxygen uptake by *Meloidogyne incognita* (Kaplan et al., 1980). In addition, this compound protects the roots from fungal attacks (*Macrophomina phaseolina, Phytophthora soyae, Fusarium solani f.*sp. *glycines* (Lygin et al., 2013; Lozovaya et al., 2004). The increase in glyceollin I, II, and III in both cultivars during the 56 days evaluation may be acting as a defense response against *P. brachyurus* invasion. This response in the BRS 7380 cultivar occurred faster and more intensely until 21 days, resulting in a higher concentration of glyceollin I, II, and III than infected MG/BR 46. Furthermore, at the end of the evaluation (56 days), infected BRS7380 stood out for its high concentration of glyceollin III. These responses may be essential for the successful defense of the cultivar BRS 7380 against the root lesion nematode. The increase in phytoalexin glyceollin was also observed in a soybean cultivar resistant to the cyst nematode after infection (Huang & Barker, 1991) and in soybean infected with *P. penetrans* after treatment with N-3-oxo-tetradecanoyl-Lhomoserin lactone, there was an accumulation of glyceollin in the roots (Adss et al., 2021).

The cultivar MG/BR 46 showed superior levels of naringenin related to BRS 7380 when infected by the nematode. This compound did not show nematicidal activity in the treatments of the specimens for 24h in the *in vitro* test. Wutys et al. (2006) also do not observe any effect on motility and chemotaxis of *P. penetras* when treated with naringenin. However, plants that absorbed this flavonoid at a concentration of 50 µg mL^{-1} and were infected by *P. brachyurus* showed a reduction in the total population by 51%, indicating that defense pathways can be activated when this compound is in higher concentration. Despite of lower levels in the BRS 7380 genotype, naringenin may be being used as a precursor for synthesis of others flavonoids. In the cultivar MG/BR 46, the conversion of naringenin to naringin resulted in a higher concentration of this product in infected plants. However, there is still no information on the function of this compound in roots.

Naringenin is also used for the synthesis of genistein. This flavonoid increased in soybean cultivars infected by *Heterodera glycines* (Kennedy et al., 1999). Ma et al., 2020 found that this flavonoid at a concentration of 50 µg mL^{-1} affects the physiology of *H. Glycines*, which results in the reduction of the reproduction rate. However, for *P. brachyurus*, this effect was not observed when treating the specimens for 24h with genistein (50 µg mL^{-1}) since the total population did not differ from the control population. On the other hand, in soybean plants that absorbed genistein, there was a reduction in the reproduction of *P. brachyurus*. In addition, the cultivar BRS 7380, which had a lower reproduction of this nematode, contained a higher concentration of genistein in the periods of 7 and 56 days in the roots. Thus, plants with higher genistein content may impair the development of *P. brachyurus*.

In cultivar, BRS 7380, part of naringenin was used for luteolin synthesis, which remained in higher concentration in this cultivar when infected by the nematode during the evaluation period of 56 days. The role of this compound in this interaction is still unknown. However, luteolin's antioxidant activity and its glycosides have been associated with their ability to eliminate reactive oxygen species (ROS) and nitrogen (López-Lázaro, 2009). ROS production is an important plant defense mechanism against nematodes. The plant cell releases these species in the apoplast region and simultaneously activates its antioxidant pathway to protect the plant cell from this oxidative damage (Rahaman et al., 2020; Gillet et al., 2017). Therefore, this increase of luteolin and its glycosylated form (21 days) in the cultivar with lower nematode reproduction may be a protective response to plant cells by activating antioxidant pathways.

5. Conclusion

The cultivar BRS 7380 showed a lower reproduction factor for *P. brachyurus* than MG/BR 46. The contrasting genotypes were used to identify changes in the concentration of metabolites in soybean cultivars (MG/BR 46 and BRS 7380) when infected by *P. brachyurus*, which involved different biochemical pathways in the defense system. IAA levels increased in response to infection by *P. brachyurus*, confirming that this phytohormone is important to the life cycle of the root lesion nematodes. The levels of IAA were similar between genotypes. Otherwise, the SA level was higher in the BRS 7380 cultivar at 56 days, indicating that SA-dependent cascades may be important for defense. The metabolic profile of soybean genotypes indicated that the class of flavonoid compounds might be necessary for the defense response. These compounds are essential for synthesizing phytoalexins, which were synthesized in higher concentrations in BRS 7380 in the presence of *P. brachyurus*. Thus, this dysregulated pathway may be involved in the molecular mechanism contributing to the lower reproduction of the nematode in the cultivar BRS 7380. It was observed that responses of increased defense-related compounds occurred predominantly in the cultivar with lower nematode reproduction. Therefore the metabolic profile analyzes indicate defense cascades acting in soybean plants to reduce the nematode infection.

6. Supplementary material

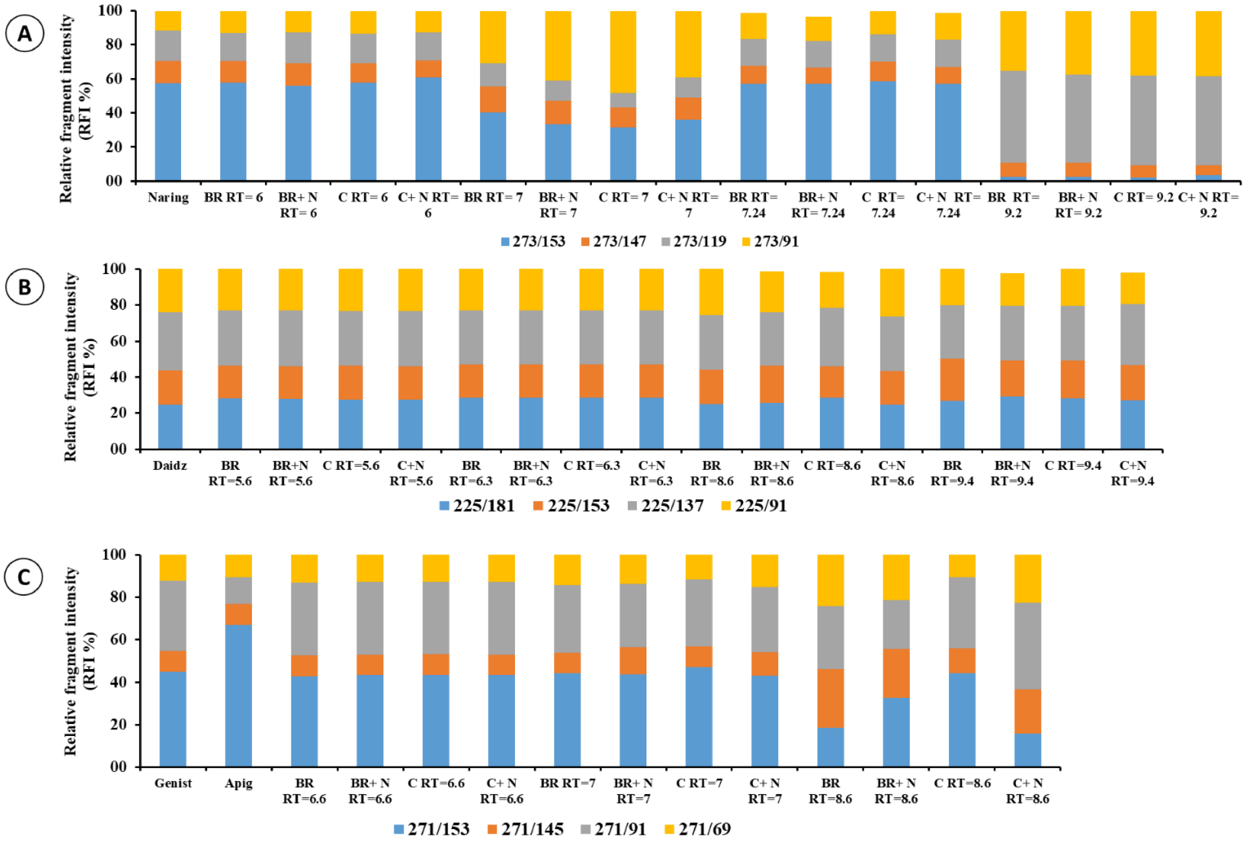

Figure S1: Nontarget analysis of flavonoids (A) Naringenin, (B) Daidzein and (C) Genistein-Apigenin) from soybean roots MG/BR 46 and BRS 7380 genotypes in the presence or absence of *Pratylenchus brachyurus* after 7 days of nematode infection. Compounds detected to each genotype, the retention times (RT) observed and their FRI% (fragment relative intensity) calculated. The compounds that share the same FRI% patterns related to standard were considered as belonging to a glycoconjugate of a certain class.

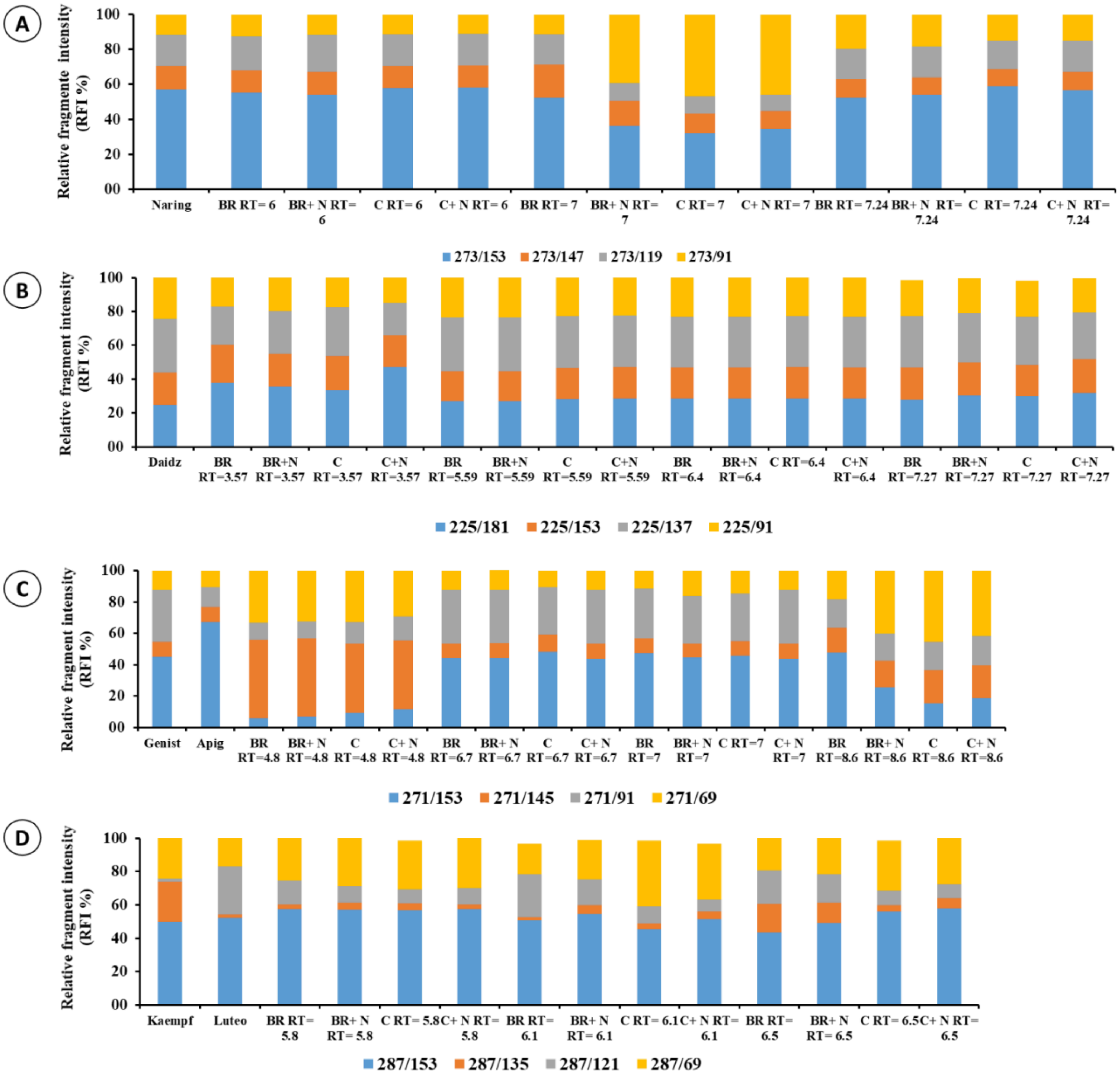

Figure S2: Nontarget analysis of flavonoids ((A) Naringenin, (B) Daidzein, (C) Genistein-Apigenin and (D) Kaempherol- Luteolin) from soybean roots MG/BR 46 and BRS 7380 genotypes in the presence or absence of *Pratylenchus brachyurus* after 21 days of nematode infection. Compounds detected to each genotype, the retention times (RT) observed and their FRI% (fragment relative intensity) calculated. The compounds that share the same FRI% patterns related to standard were considered as belonging to a glycoconjugate of a certain class.

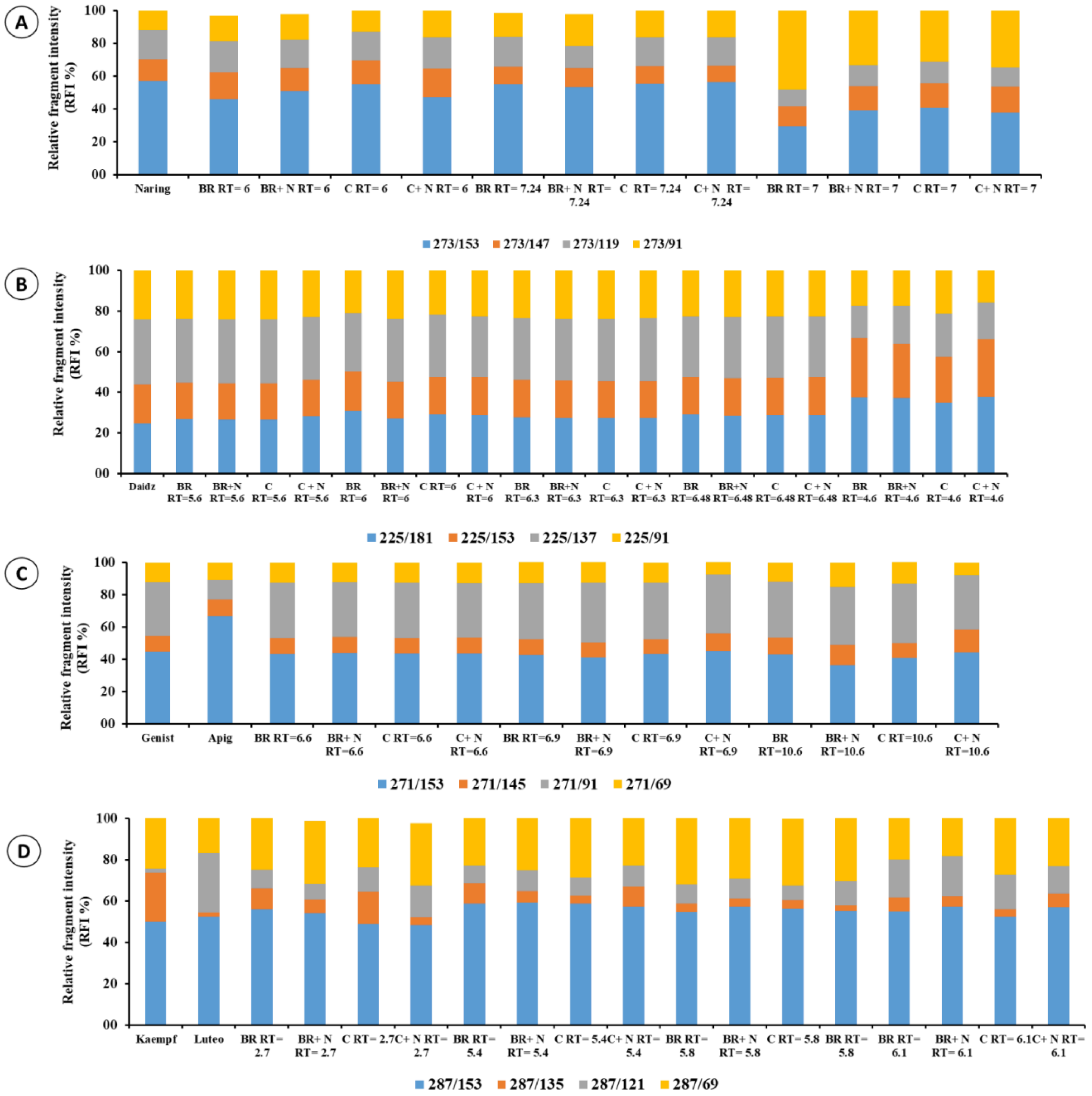

Figure S3: Nontarget analysis of flavonoids ((A) Naringenin, (B) Daidzein, (C) Genistein-Apigenin and (D) Kaempherol- Luteolin) from soybean roots MG/BR 46 and BRS 7380 genotypes in the presence or absence of *Pratylenchus brachyurus* after 56 days of nematode infection. Compounds detected to each genotype, the retention times (RT) observed and their FRI% (fragment relative intensity) calculated. The compounds that share the same FRI% patterns related to standard were considered as belonging to a glycoconjugate of a certain class.

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CAPÍTULO 4

RESUMO

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, junho de 2022. **Inoculação de *Pochonia chlamydosporia* promove redução diferencial na população do nematoide *Pratylenchus brachyurus* e alteração nos perfis de metabólitos dos genótipos de soja.** Orientador: Humberto Josué de Oliveira Ramos. Coorientadores: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes e Maria Goreti de Almeida Oliveira.

Pratylenchus é um dos gêneros de nematoides parasitas de plantas que mais causam perdas na agricultura. Em soja, *Pratylenchus brachyurus*, é responsável por grandes perdas na produtividade, pois as cultivares apresentaram baixa resistência ao ataque do nematoide. A combinação de métodos de manejo pode contribuir para o controle desse nematoide, como o uso de microrganismos nematófagos, como *P. chlamydosporia* e cultivares com menor fator de reprodução para esse nematoide. Neste trabalho, avaliou-se a resposta de defesa das plantas nos dois genótipos de soja, BRS 7380, que apresenta fator de reprodução para *Pratylenchus* inferior ao MG/BR 46. A inoculação de *P. chlamydosporia* foi eficiente no controle de nematoides parasitas de plantas. No entanto, esse efeito foi dependente do genótipo, pois a presença do fungo reduziu o número de nematoides nas raízes do genótipo MG/BR 46 e aumentou para o genótipo BRS 7380. Os perfis fitohormonais também foram distintos entre os genótipos, indicando diferentes cascatas de resposta das plantas sob colonização fúngica e ataque de nematoides. Hormônios, flavonoides, fitoalexinas (coumestrol, gliceolina I, II, III e medicarpina), fenólicos totais e lignina também foram alterados em raízes infectadas por *P. brachyurus* e na presença de *P. chlamydosporia*. A presença do fungo induziu alterações semelhantes nas duas cultivares, mas a maior concentração de alguns compostos na cultivar BRS 7380 pode ter afetado a colonização fúngica, como o maior teor de lignina. A maior resposta de coumestrol e gliceolina na MG/BR 46 aos 56 dias com o fungo pode ter contribuído para o controle nesta cultivar. As interações neste patossistema são altamente complexas e pode ser necessário reduzir alguns compostos de defesa para ocorrer a colonização fúngica. No entanto, isso não implica necessariamente em maior suscetibilidade ao patógeno, pois o fungo pode ativar outros mecanismos de defesa para proteger a planta, além de se alimentar dos ovos do nematoide.

Palavras chave: *Pochonia chlamydosporia. Pratylenchus brachyurus.* Flavonoides. Fitolalexinas.

ABSTRACT

GOUVEIA, Angélica de Souza, D.Sc., Universidade Federal de Viçosa, June, 2022. **Inoculation of *Pochonia chlamydosporia* promotes differential reduction in the population of the nematode *Pratylenchus brachyurus* and changes in metabolite profiles of soybean genotypes.** Advisor: Humberto Josué de Oliveira Ramos. Co-advisors: Leandro Grassi de Freitas, Thalita Suelen Avelar Monteiro, Virgílio Adriano Pereira Loriato, Elizabeth Pacheco Batista Fontes and Maria Goreti de Almeida Oliveira.

Pratylenchus is one of the plant-parasitic genera of nematodes that causes most losses in the agriculture. In soybean, *Pratylenchus brachyurus*, is responsible for large reductions in productivity because the cultivars show low resistance to this nematode parasitism. The combination of management methods can contribute to the control of this nematode, like the use of nematophagous microorganisms such as *P. chlamydosporia* and cultivars with low reproduction factors for this nematode. In this work, it was evaluated the plant defense response in the two soybean genotypes, BRS 7380 with a lower reproduction factor for *Pratylenchus* than the MG/BR 46 cultivar. Inoculation of *P. chlamydosporia* was efficient for the control of the plant parasitic nematode. However, this effect was genotype-dependent because the presence of the fungus reduced the nematode number in the roots of the MG/BR 46 and increased in the BRS 7380 genotype. The phytohormonal profiles were also distinct between genotypes, indicating different response cascades of the plants under fungal colonization and nematode attack. Hormones, flavonoids, phytoalexins (coumestrol, glyceollin I, II, III and medicarpin), total phenolics and lignin were also changed in roots infected by *P. brachyurus* and in the presence of *P. chlamydosporia*. The presence of the fungus induced similar changes in both cultivars, but the higher concentration of some compounds in the BRS 7380 cultivar may have affected the fungal colonization, such as the higher lignin content. The greater response of coumestrol and glyceolin in MG/BR 46 at 56 days with the fungus may have contributed to the control in this cultivar. The interactions in this pathosystem are highly complex, and it may be necessary to reduce some defense compounds for fungal colonization. However, this does not necessarily imply greater susceptibility to the pathogen, as the fungus can activate other defense mechanisms to protect the plant besides the direct effect of the fungus wich kills nematode eggs.

Keywords: *Pochonia chlamydosporia. Pratylenchus brachyurus.* Flavonoids. Phytolalexins.

1. Introduction

The genus *Pratylenchus* is represented by migratory endoparasitic nematodes that move and feed intercellularly through the root cortex, not stablishing a specific nursing site. The parasitism of this nematode causes root lesions, which facilitate the infection of other pathogens, resulting in necrotic roots. Consequently, the absorption of water and nutrients is affected, compromising productivity (Fosu-Nyarko & Jones, 2016). *Pratylenchus* is one of the most important plant parasitic nematode genera in terms of economic impact on agriculture and has a wide range of hosts, such as wheat, barley, chickpeas, sugar cane, and soybeans (Jones & Fosu-Nyarko, 2014).

For soybean, resistance genes that could be incorporated into other genotypes contributing to the control of *P. brachyurus* have not yet been detected. In wheat, the resistance inheritance of *P. thornei* is polygenic and additive, meaning that numerous compounds may be responsible for the defense responses, which makes it challenging to make resistant wheat varieties available (Sheedy & Thompson, 2009; Sheedy et al., 2012; Rahaman et al., 2021). Another control alternative is the use of microorganisms applied to the soil. The interaction established with the root and direct mechanisms on the nematode contribute to reducing the final population.

In the soybean cultivar (BRSGO Caiapônia) the addition of *T. harzianum* and *T. asperellum* separately efficiently controlled *P. brachyurus* after 60 days (Oliveira et al., 2021). The control efficiency of *T. harzianum* and *Purpureocillium lilacinum* for *P. brachyurus* was proven in a greenhouse and field test (Dias-Arieira et al., 2018). *P. chlamydosporia* isolates (Pc-3, Pc-10, Pc-35) reduced the total population of *P. brachyurus* by 43.7% in soybean (M9350) compared to the control treatment (Pacheco et al., 2020). However, the mechanisms of plant defense promoted by these microorganisms to control the root lesion nematode have not yet been investigated.

Thus, this work evaluated the capability of *P. chlamydosporia* to control *P. brachyurus* and compared the plant defense responses from two cultivars, BRS 7380 with a lower reproduction factor to *Pratylecnhus* than MG/BR 46. The biochemical changes in the root infected by the nematode were identified using metabolomics tools. In addition to identifying changes in biochemical pathways due to the presence of the biological control fungus, it is interesting to investigate the combination of management tools, such as microorganisms and cultivars that allow less nematode reproduction, as alternatives for a more effective control of this pathogen.

2. Methodology

2.1 Nematodes, fungus and plants

The *P. brachyurus* inoculum was multiplied in soybean plant in a greenhouse, and the nematode extraction was carried out by the method proposed by Coolen and D'Herde (1972). The quantification of the number of nematodes was performed in a Peters chamber. The fungus *P. chlamydosporia* (Pc-10) belongs to the collection of the Laboratory of Biological Control of Phytonematodes of the Federal University of Viçosa, Minas Gerais state, Brazil. This fungus was cultivated in autoclaved rice, and the growth was maintained for 21 days at 27 ºC to obtain chlamydospores. After this time interval, these structures were recovered with aqueous extraction and filtered with gauze. Chlamydospore suspension was quantified in a Neubauer chamber. In this study, the soybean cultivars MG/BR 46 (Conquista) and BRS 7380 were used, and the seeds were disinfected with gaseous chlorine.

2.2 Experiment in a greenhouse

The greenhouse experiment was carried out with the following treatments: MG/BR 46 + *P.brachyurus*; MG/BR 46 + *P.brachyurus* + *P. chlamydosporia*; BRS 7380 + *P.brachyurus* and BRS 7380 + *P.brachyurus* + *P. chlamydosporia*. In each 2L plastic pot containing sterilized substrate (C horizon clayey soil and washed sand (1:1)) 5000 chlamydospores/g of soil and one soybean seed was added. After 12 days, 500 specimens of *P. brachyurus* were inoculated per soybean seedling. The experiment was evaluated in three periods, 7, 21, and 56 days after nematode inoculation, and the roots were collected and immediately frozen with liquid nitrogen for biochemical analysis.

In the 56 days, the specimens of *P. brachyurus* were extracted by the method proposed by Coolen and D'Herde (1972) and quantified in an optical microscope. The experimental design was randomized and three replicates were used for biochemical analysis, each replicate formed by a pool of 3 plants. For the evaluation of nematode reproduction, 7 replicates were used. The control experiment performed by the fungus was repeated under the same conditions described above. However, the evaluation was made 80 days after the nematode inoculation. In addition to nematode reproduction, fresh root mass, dry shoot mass, and the number of pods per plant were evaluated.

2.3 Biochemical analyzes of the root

2.3.1 Total Phenolics and Lignin

The total phenolic compounds were extracted from 100 mg of root of each treatment (MG/BR 46 + *P. brachyurus*; MG/BR 46 + *P.brachyurus* + *P. chlamydosporia*; BRS 7380 + *P. brachyurus* and BRS 7380 + *P. brachyurus* + *P. chlamydosporia*) using 80% methanol (v/v). Centrifugation was performed after 12 hours of contact between the extracting solution and the samples. The recovered supernatant was used to analyze total phenolics using the Folin-Ciocalteu method (Makkar et al., 1993). The standard curve of gallic acid (mg/L) was used to quantify the total phenolics, and the final result was expressed in mg of gallic acid equivalents per gram of fresh root (mg GAE/g).

After the samples were centrifuged, the supernatant was used to quantify phenolics, and the pellet was used to determine the lignin content by the thioglycolic acid method (Campbell et al., 1992). Lignin was quantified as absorbance values (280nm) per gram of dry root (A280nm g^{-1}).

2.3.2 Evaluation of compounds by LC/MS

Hormones, flavonoids, and phytoalexins were extracted from 200 mg of root with 700 µL of extractor solution (methanol, isopropanol, and acetic acid solution (20: 79: 1), according to Vital (2019). The metabolites were separated by chromatography (UHPLC-Agilent), using a C18 column (50 mm x 1.0 mm ID, 1.7 µm particle, and 300 A), coupled on-line to the triple quadrupole mass spectrometer (QqQ). This equipment was operated in MRM (Multiple Reaction Monitoring) mode. The spectra referring to hormones and phytoalexins were analyzed using the Skyline software, which allowed the quantitative analysis of the compounds in the root. The flavonoid profile was performed according to the methodology of Gómez et al. (2018).

2.4 Statistical analysis

The means and standard deviations of the results were calculated, and the data were submitted to analysis of variance (ANOVA), followed by Duncan's test with a significance of 5%, using the R version 3.6.0 program.

3. Results

3.1 Reproduction of the root-lesion nematode in soybean inoculated with *P. chlamydosporia*

The ability of *P. chlamydosporia* to reduce the *P. brachyurus* in roots was evaluated for two cultivars, BRS 7380 and MG/BR 46. The cultivar BRS 7380 presents lower reproduction rates of this root-lesion nematode (Gouveia et al., 2022c) than MG/BR 46. In accordance, the total population of nematodes was lower in BRS 7380 than in cultivar MG/BR 46. However, the nematode population was increased in plants from the BRS 7380 under infection by biological control fungus (**Figure 1**). On the other hand, in the MG/BR 46 cultivar, the presence of the fungus effectively reduced the nematode population. This experiment was repeated for a longer interval (80 days), and it was confirmed that the biological control fungus could have different behaviors according to the soybean genotypes (**Figure 1**).

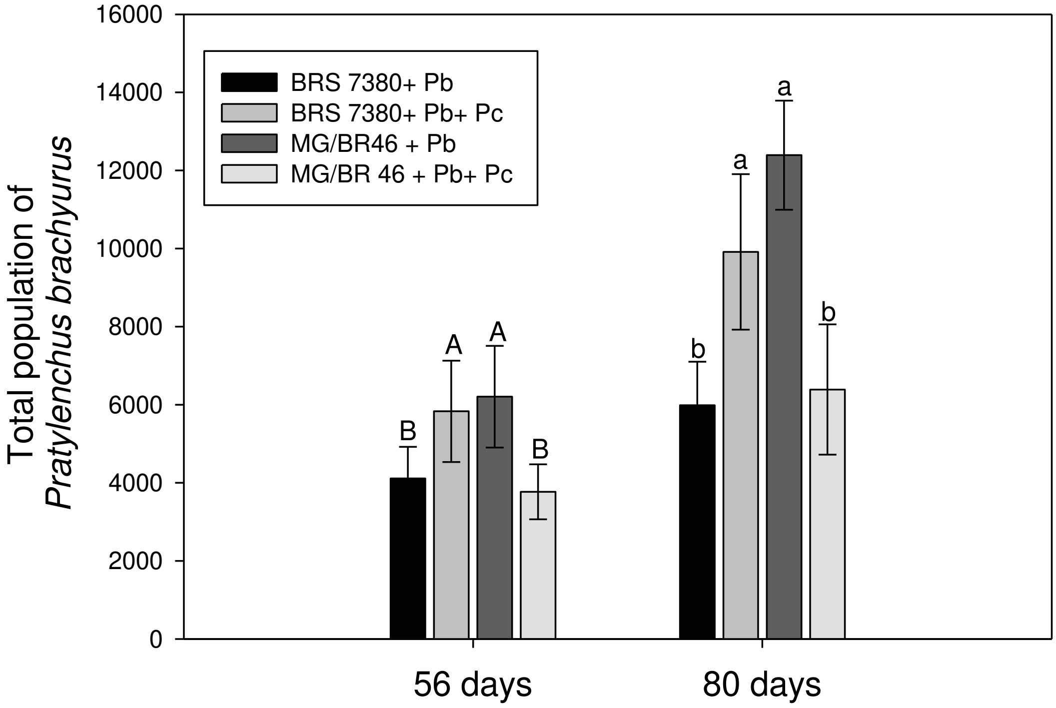

Figure 1: The population number of *Pratylenchus brachyurus* (Pb) specimens in soybean cultivars (MG/BR 46 and BRS 7380) inoculated or not with *Pochonia chlamydosporia* (Pc) in two evaluation periods (56 and 80 days). The treatment mean followed by capital letter differs from Duncan's test ($p < 0.05$) after 56 days of the addition of nematodes, and the mean followed by lower case letter differs from Duncan's test ($p < 0.05$) after 80 days of the addition of the nematodes. Bars represent the standard deviation (n =7).

3.2 Development of plants inoculated with *Pochonia chlamydosporia* and infected with *Pratylenchus brachyurus*

The interaction of *P. chlamydosporia* with the cultivar BRS 7380 also negatively affected plant development. This interaction resulted in lower dry shoot mass and a reduced number of pods per plant (**Figures 2A and 2B**). While the presence of the fungus in the MG/BR 46 cultivar provided an increase in the dry shoot mass and a significant increase in the number of pods (**Figure 2A and 2B**). These results are in accordance with the effect of the fungal colonization in the nematode infection of soybean genotypes.

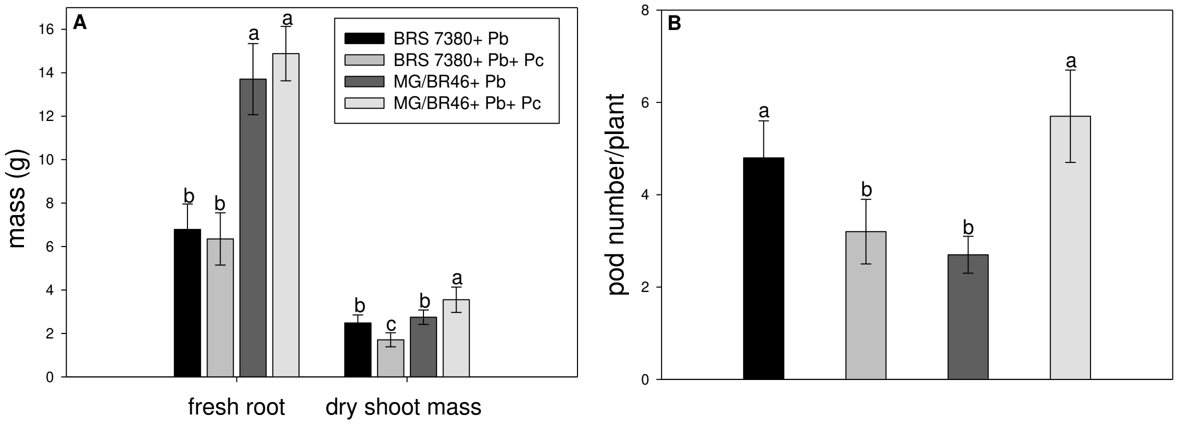

Figure 2: **A)** Fresh root mass and dry shoot mass and **B)** the number of soybean pods referring to cultivars MG/BR 46 and BRS 7380 infected by *Pratylenchus brachyurus* and inoculated or not with *Pochonia chlamydosporia* (Pc) after 80 days. According to Duncan's test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$). Bars represent the standard deviation (n =7).

3.3 Biochemical analyzes in the root

3.3.1 Hormonal analysis

The interaction of *P. chlamydosporia* with the roots of cultivars infected by *P. brachyurus* promote changes in phytohormonal profiles. In the evaluation after 7 days of the inoculation of the nematode, the fungus reduced salicylic acid (SA) in the cultivar MG/BR 46. In contrast, after 56 days, the fungus caused an increase in the production of SA in the BRS 7380 (**Figure 3A**). The production of jasmonic acid (JA) was reduced due to the fungus in both cultivars at 7 and 21 days. In the 56 days evaluation, the fungus induced a JA reduction only in the MG/BR 46 cultivar (**Figure 3B**).

The fungus's interaction with the cultivars' roots induced similar changes concerning the IAA hormone, which was reduced at 7 and 56 days under co-inoculation conditions (**Figure 3C**). For the ABA hormone, the fungus maintained a reduction in the hormonal level, mainly in the MG/BR 46 cultivar, which the treatment with the fungus reduced the concentration of this hormone in the roots at 7 and 21 days. However, in the 56 days evaluation, there was an increase in ABA in this treatment. For the cultivar BRS 7380, significant change in the level of ABA due to the fungus occurred only at 56 days, which resulted in the reduction of this hormone (**Figure 3D**). Thus, ABA levels correlated with alterations in infection pattners, being that increase of the levels under fungal infection in the MG/BR 46 may be acting in balance to the reduction of the SA/JA levels after 56 days of intection.

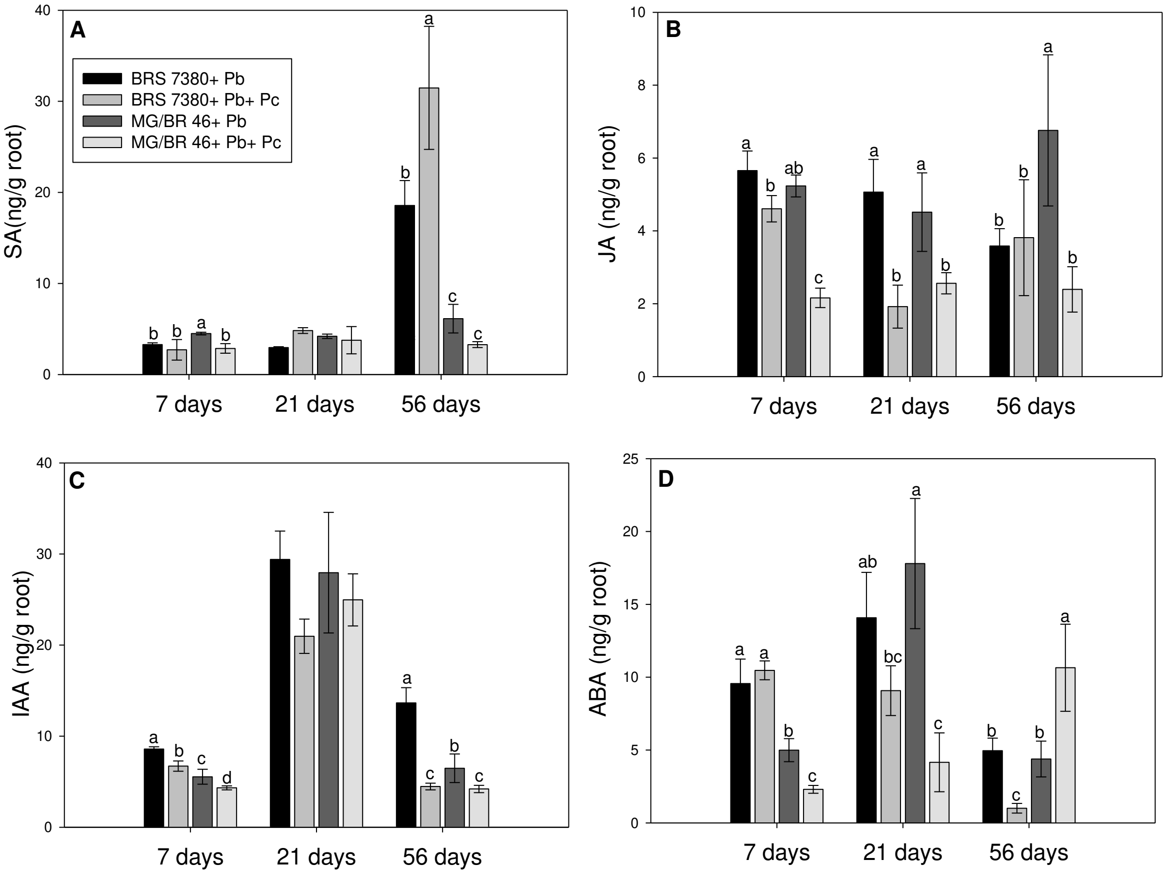

Figure 3: Quantification of phytohormones in soybean roots of cultivars MG/BR 46 and BRS 7380 infected by *Pratylenchus brachyurus* and inoculated or not with *Pochonia chlamydosporia* (Pc) **A)** Salicylic acid (SA); **B)** Jasmonic acid (JA); **C)** Indole-3-acetic acid (IAA) and **D)** Abscisic acid (ABA). According to Duncan's test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$) in the respective evaluation period.

3.3.2 Total phenolics and lignin

The quantification of total phenolics showed that the fungal inoculation differently interfered in its levels, according to the period of interaction and the genotypes. In cultivar MG/BR 46, at 7 days, the fungus induced the reduction of phenolic compounds, and at 21 days, the fungus increased these compounds, which may be important in the control of the nematode. In the cultivar BRS 7380, the presence of the fungus in the infected roots caused an increase in total phenolic compounds at 21 days and a reduction in the evaluation of 56 days (**Figure 4A**).

The interaction established between the cultivar BRS 7380 infected by *P. brachyurus* and with *P. chlamydosporia* induced an increase in lignin synthesis in this cultivar at 7 and 21 days. While in the MG/BR 46 cultivar, the presence of the fungus did not cause significant changes in the quantification of lignin (**Figure 4B).** The lignin levels did not correlate with the infection behavior of nematodes under fungal infection (**Figure 1**).

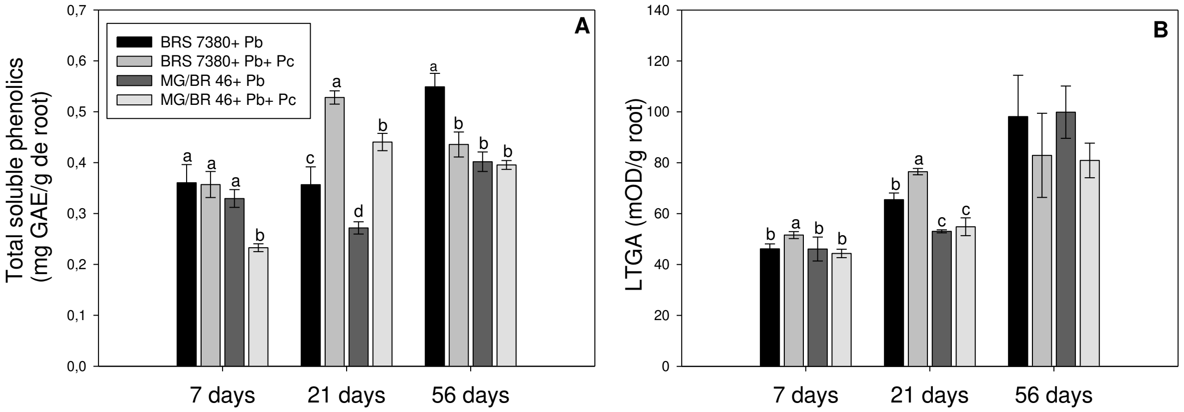

Figure 4: A) The concentration of total soluble phenolic compounds and **B)** Concentration of lignin-thioglycolic acid derivatives (LTGA) in soybean roots of cultivars MG/BR 46 and BRS 7380 infected *by Pratylenchus brachyurus* and inoculated or not with *Pochonia chlamydosporia* (Pc). According to Duncan's test after ANOVA, different letters indicate a significant difference between treatments ($p < 0.05$) in the respective evaluation period. GAE: Gallic Acid Equivalent

3.3.3 Analysis of flavonoids in soybean root

Phenylpropanoid pathway have been responsive to infections by fungus and nematodes. Thus, the root extracts were submitted to a metabolomic approach by LC/MS to generate the dysregulated profiles of flavonoids. Applying the approaches described by Gomez et al., 2018, it was possible to identify and quantify the aglycones and glycoconjugates for daidzein, genistein, naringin and luteolin in the different treatments represented, as indicated in the heatmap (**Figure 5**) and described in accordance with the retention times: naringenin (RT= 6 min), daidzein (RT=5.6; 6.3; 8.6 min) and genistein (RT= 6.6; 7 min) at 7 days. For 21 days, naringenin (RT=6 min), daidzein (RT=5.6; 6.5 min), genistein (RT=7 min) and luteolin (RT=5.8 min) were identified. At 56 days, daidzein (RT=5.6; 6, 6.3; 6.5 min), naringenin (RT=6 min), luteolin (RT=5.8 min), and genistein (RT= 7 min) were detected in the samples. Then, in these retention times, the m/z value of the precursor ion was manually verified in the XIC (extracted ion chromatogram), and its identification was carried out from searches in the Mass Bank. With this method, the compounds present in **Table 2** were identified.

Table 2: Identification of glycosylated flavonoids present in the roots of cultivars BRS 7380 and MG/BR 46 infected by *Pratylenchus brachyurus* and inoculated with *Pochonia chlamydosporia* in the three evaluation periods (7, 21 and 56 days).

Flavonoid Class	Precursor	RT (min)	Conjugate (m/z)	Mass	Relative Abundance in XIC (%)	Database
7 days						
Daidzein	255	5.6	417.2	416.11	100	Daidzein-7-O-glucoside
	255	6.3	503.2	502.11	70.99	Daidzein 7-O-glucoside-6''-O-malonate
	255	8.6	503	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
Genistein	271	6.6	433.1	432.1	100	Genistein 7-O-glucoside
	271	7	519.3	518.1	100	Genistein 7-O-glucoside-6''-O-malonate
Naringenin	273	6	581.2	580.17	100	Naringin
21 days						
Daidzein	255	5.6	417.1	416.11	100	Daidzein-7-O-glucoside
	255	6.5	503	502.11	100	Daidzein 7-O-glucoside-6''-O-malonate
Genistein	271	7	519.4	518.1	55.01	Genistein 7-O-glucoside-6''-O-malonate
Luteolin	287	5.8	448.8	448.1	100	Luteolin-7-O-glucoside
Naringenin	273	6	581.2		100	Naringin
56 days						
Daidzein	255	5.6	417.2	416.11	100	Daidzein-7-O-glucoside

	255	6	503.2	502.11	100	Daidzein 7-O-glucoside-6"-O-malonate
	255	6.3	503.2	502.11	100	Daidzein 7-O-glucoside-6"-O-malonate
	255	6.5	503.3	502.11	100	Daidzein 7-O-glucoside-6"-O-malonate
Genistein	271	7	519.4	518.1	100	Genistein 7-O-glucoside-6"-O-malonate
Luteolin	287	5.8	448.9	448.1	100	Luteolin-7-O-glucoside
Naringenin	273	6	581.2	580.1	100	Naringin

The presence of the biological control fungus altered ($p < 0.05$) the concentration of some of these compounds similarly in both cultivars infected by *P. brachyurus*. However, for the compound genistein, the presence of the fungus caused an increase only in the cultivar BRS 7380 infected at 7 days. In the same evaluation period, the fungus interfered differently in the concentration of daidzein 7-O-glucoside, which increased in the cultivar BRS 7380, and there was no change in the cultivar MG/BR 46.

The roots of the MG/BR 46 cultivar with *P. chlamydosporia* have significatively lower amounts of genistein, daidzein, genistein7-O-glucoside, daidzein7-O-glucoside, and daidzein 7-O-glucoside-6"-O-malonate compared to the cultivar BRS 7380 under fungal inoculation. In the 21 days evaluation, the fungus also similarly induced changes in some flavonoids in the two infected cultivars. However, there was no statistical difference between cultivars BRS 7380 with the fungus and MG/BR 46 with the fungus for luteolin, naringenin, genistein, genistein 7-O-glucoside-6"-O-malonate, daidzein, and daidzein 7-O-glucoside. At 56 days, the alterations caused by the presence of the fungus maintained similar modification patterns for both cultivars. However, for the compounds luteolin, genistein, genistein-7-O-glucoside-6"-O-malonate and daidzein, there was no significant difference between treatments BRS 7380 with the fungus and MG/BR 46 with the fungus.

Some of the flavonoids identified are precursors for synthesizing phytoalexins that directly contribute to plant defense responses. Therefore, the alteration caused by the biological control fungus in the production of coumestrol, glyceollin (I, II, III), and medicarpin was investigated in the roots of cultivars infected by *P. brachyurus* (**Figure 5A**). At 7 days the infected BRS 7380 cultivar contained a high concentration of coumestrol, but the presence of the fungus induced the reduction of this compound. While in the susceptible cultivar, there were no changes due to the presence of the fungus. This cultivar already had a lower concentration of coumestrol compared to BRS 7380. For glyceollin, I, and III, the fungus did not change the concentration of these compounds in both cultivars. However, for glyceollin II in the MG/BR 46 cultivar, the fungus induced an increase in the concentration of the compound. For medicarpin, there was an increase in both cultivars due to the presence of the fungus.

The presence of the fungus after 21 days of nematode infection resulted in a reduction of some phytoalexins (**Figure 5B**), such as glyceollin I and II, for both cultivars. As for glyceollin III and medicarpin, there was a reduction only in the cultivar MG/BR 46. Coumestrol levels did not change due to fungal inoculation in both cultivars after 21 days of nematode infection.

However, the production of this compound was modified at last evaluation time (**Figure 5C**). At 56 days, the presence of the fungus induced an increase in coumestrol in MG/BR 46, while in cultivar BRS 7380, there was a reduction of this compound. At that moment, the presence of the fungus also induced an increase in glyceollin I and II in the MG/BR 46 cultivar. There were no changes in concentration for glyceollin III and medicarpin due to the presence of the fungus in both cultivars.

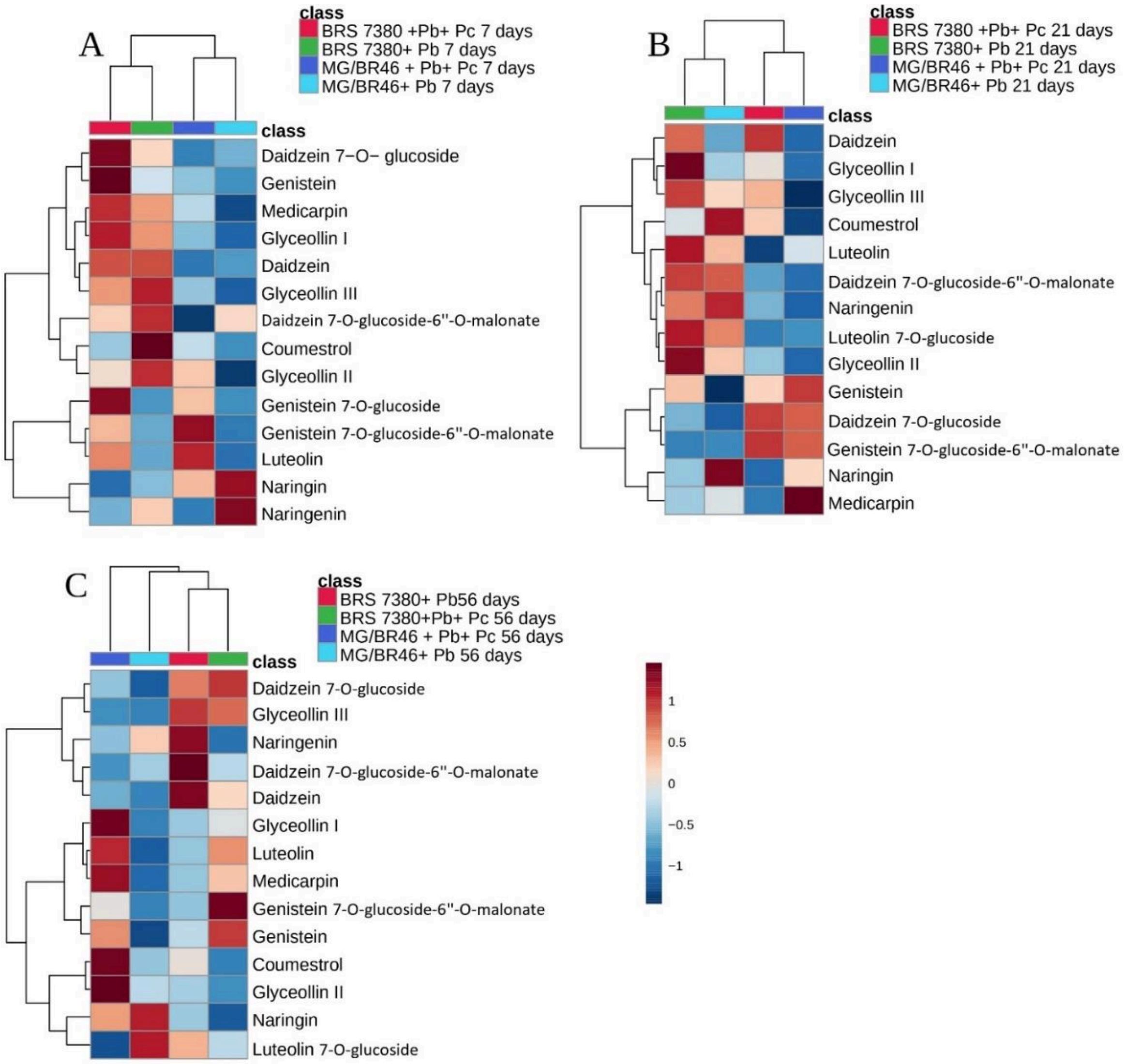

Figure 5: Cluster analysis by the Heatmap method of flavonoids characterized in soybean roots of genotypes MG/BR 46 and BRS 7380 infected by *Pratylenchus brachyurus* in the absence or presence of *Pochonia chlamydosporia* **(A)** 7 days **(B)** 21 days and **(C)** 56 days after nematode infection. The graph represents the differences in the abundance of metabolites analyzed by LC-MS in response to treatments, with the blue color indicating reduction and red increase.

4. Discussion

P. chlamydosporia has been used to control root-knot nematode, which may involve direct and indirect mechanisms to reduce reproductive capacity (Yi et al., 2021). This fungus also efficiently controlled *P. brachyurus* in soybean, cotton, and corn (Pacheco et al., 2020; Costa et al., 2015). However, mechanisms used by the fungus to control this nematode are still unknown and may involve changes in the plant metabolism, as verified by root-knot nematodes (Gouveia et al., 2022b). Thus, biochemical alterations from two soybean genotypes triggered by *P. chlamydosporia* inoculation were evaluated during the infection by the root-lesion nematode *P. brachyurus*. The cultivar BRS 7380 has shown a lower reproduction factor for the root lesion nematode (*Pratylenchus* spp) than the cultivar MG/BR 46, which is considered susceptible to this nematode (Gouveia et al., 2022c).

When conducting the experiment with *P. brachyurus* in the cultivar BRS 7380, the final population of nematodes was lower than in the susceptible cultivar, confirming its low reproduction factor. However, inoculation with *P. chlamydosporia* in the cultivar BRS 7380 promoted an increase in the population of *P. brachyurus*. On the other hand, in the MG/BR 46 cultivar, the fungus efficiently controlled *P. brachyurus*, resulting in a smaller population. Thus, the effect of the fungal inoculation was genotype-dependent. A similar result occurred in the study by Oliveira et al. (2021) in the soybean cultivar, BRSGO Caiapônia, the addition of *Trichoderma harzianum*, and *Trichoderma asperellum* resulted in *P. brachyurus* control. In the BRS 8560RR cultivar, the same treatments did not reduce the population of *P. brachyurus.*

Notably, the fungal colonization triggered a distinct metabolic response for the soybean genotypes under nematode attack. The presence of the microorganism possibly disrupted or activated biochemical pathways that favored or reduced the development of the nematode. This increase in the population of nematodes may have contributed to the reduction in dry shoot mass and the number of pods per plant. After identifying a relationship between the reduction or increase of the nematode population performed by *P. chlamydosporia* according to the cultivar, possible mechanisms were investigated to explain the behavior observed for the cultivars BRS 7380 and MG/BR 46.

Phytohormones are related to different stages of nematode parasitism in plants resulting in a complex network of synergistic effects and antagonistic interactions. Salicylic acid regulates defense responses against pathogens by inducing pathogenesis-related genes such as PR-1 and PR-7 (Glazebrook, 2005). Plants are generally more susceptible to root-knot nematode when there is a lower concentration of salicylic acid (Wubben et al., 2008; Nahar et al., 2011). The increases of the SA levels by fungal inoculation in the BRS 7380 did not culminate in the activation of the plant defense cascades in this genotype and did not correlate with the increase in the number of the specimens observed in the roots from the BRS 7380 genotype. In contrast, the SA levels were markable reduced by fungal colonization in the MG/BR 46 plants, and it was in accordance with the reduction of the nematode number in its roots. Thus, these results confirm that SA-dependent cascades may be important for root-lesion nematode infection in soybean plants. Otherwise, a balance between SA/JA levels may be acting to reduce the nematode infection under fungal root colonization in the MG/BR 46 plants.

In the association of a fungus with roots, plant hormones can be important in communication and establishing symbiosis. In addition to regulating plant growth and development, hormones provide responses to control the colonization of beneficial microorganisms (Yan et al., 2019). Tomato and *A. thaliana* roots colonized by *P. chlamydosporia* reduce jasmonic acid production, possibly a necessary mechanism for fungal colonization (Zavala-Gonzalez et al., 2016; Gouveia et al., 2022). A similar result occurred in the MG/BR 46 cultivar, in which the fungus reduced the JA concentration in the three evaluated periods. At 7 days, the highest concentration of JA in the root of the cultivar BRS 7380 with the fungus may have impaired fungal colonization, affecting the ability to control nematodes.

For the auxin hormone, the presence of the fungus in the infected plants induced the same responses in the cultivars since there was a reduction of this hormone at 7 and 56 days. Different studies with other species of *Pratylenchus* have showed biochemical changes related to auxin (Channale et al., 2021; Vieira et al., 2019; Rahaman et al., 2020). However, the importance of this increase for different species of plants infected by different species of *Pratylenchus* is still unknown.

As in the hormonal evaluation, it is possible to verify that the presence of the fungus induced different responses over time in the cultivars for phenolic compounds and flavonoids. For the MG/BR 46 and BRS 7380 cultivars, only in the 21 days evaluation a higher concentration of total phenolics was detected due to the fungus in the infected roots. These compounds also increased in plants infected *by P. penetrans, P. coffeae, P. zeae,* and *P. thornei*, possibly a plant defense response, as the oxidation of these compounds can result in the formation of quinones that have antimicrobial activity (Acedo et al. al., 1971; Backiyarani et al., 2013; Vaganan et al., 2014; Mehta et al., 2005; Rahaman et al., 2020).

In the cultivar BRS 7380 under nematode infection, when *P. chlamydosporia* was also present, there was an increase in lignin deposition in the roots at 7 and 21 days. The increase in lignin is an important defense response in an attempt to contain the infection of pathogens, mainly for plant parasitic nematodes. Infection of pathogenic fungi such as *Verticillium dahliae* on cotton is restricted by increased tissue lignification (Xu et al., 2011). *Bacillus amyloliquefaciens*, an endophytic bacteria beneficial to the plant, resulted in an increase in lignin in the root even without the presence of a pathogen (Irizarry et al., 2017). On the other hand, the lignin content in tomato roots infected by *M. javanica* and inoculated with arbuscular mycorrhiza or *Trichoderma harzianum* was reduced in these interactions. However, both biological control agents efficiently reduced the nematode, mainly by combining the two agents. The combination of *T. harzianum* and mycorrhiza in plants infected by the nematode provided a greater reduction in lignin content than treatments using only a control agent (Nafady et al., 2022).

Possibly the increase that occurred in the BRS 7380 cultivar due to the presence of the fungus impaired its colonization, affecting the ability to nematode control. In the cultivar MG/BR 46, in which the fungus reduced the population of nematodes, there was no change in the lignin content due to the presence of the fungus. At 21 days in this cultivar, the lignin quantification was lower than in the BRS 7380, which may have favored colonization of the fungus.

Evaluating flavonoids in the roots can be important to identify plant defense mechanisms in soybean when infected by *P. brachyurus*, to obtain profiles from its free aglycones and glycosidic conjugates. When evaluating the profiles induced by *P. chlamydosporia* in the cultivars BRS 7380 and MG/BR 46 infected by *P. brachyurus*, it is possible to highlight some changes that are possibly important in this interaction. At 7 days in the cultivar MG/BR 46 with the fungus, there was a lower concentration of some compounds such as daidzein, genistein-7-O-glucoside, daidzein 7-O-glucoside, daidzein 7-O-glucoside-6''-O-malonate, concerning BRS 7380 with the fungus.

Interestingly, at 56 days, daidzein 7 O-glucoside and daidzein 7-O-glucoside-6''-O-malonate remained in lower concentration in the infected MG/BR 46 cultivar with the fungus compared to infected BRS 7380 with the fungus. Throughout the evaluation, the compound naringin was more concentrated in the roots MG/BR 46 than BRS7380 both in the presence of the fungus. The precursor of this compound, naringenin which showed highest concentration in the 56 days evaluation in MG/BR 46 with the fungus. Thus, the naringin synthesis pathway may be important for the control performed by *P. chlamydosporia* in the MG/BR 46 cultivar, and the reduction of compounds mentioned previously does not indicate an increase in susceptibility due to the control result found.

The evaluation over time allowed us to conclude greater influence of the fungus on glyceollin changes in the root after 21 days. During this period, glyceollin II was reduced in both cultivars and glyceollin III decreased only in cultivar MG/BR 46 with the fungus. For glyceollin I, the BRS 7380 cultivar has a higher amount than the MG/BR 46 cultivar, and the presence of the fungus reduced this compound in both cultivars. However, in the roots of BRS

7380 with the fungus still contained a greater amount of this compound in relation to MG /BR46 with the fungus. Thus, the lower amount of glyceollin in the cultivar MG/BR 46 may have contributed to the better performance of the fungus in this cultivar.

There are still no records on the effect of glyceollin on beneficial microorganisms, but for pathogenic fungi this phytoalexin is an important plant defense mechanism. In soybean roots with lower concentration of glyceollin, there was better development of the pathogenic fungi, *M. phaseolina*, *P.sojae,* and S. *sclerotiorum* (Lygin et al., 2010). Furthermore, the higher production of glyceollin in soybean roots may determine the plant's ability to combat *Fusarium solani f. sp. glycines* (Lozovaya et al., 2004). Therefore, the high concentration of glyceollin in the cultivar BRS 7380 may be one of the factors that interfered with the colonization of *P. chlamydosporia*, resulting in the absence of control of root lesions nematode.

During this interaction, 56 days, there is no interference of the fungus in the cultivar BRS 7380 in the production of glyceollin. However, in the MG/BR 46 cultivar that had *P. brachyurus* control with the fungus, there was an increase in glyceollin I and III production. The increase in this phytoalexin was also observed in a soybean cultivar resistant to the cyst nematode after infection (Huang & Barker, 1991) and in soybean infected by *P. penetrans* after treatment with N-3-oxo-tetradecanoyl-Lhomoserin lactone, there was the accumulation of glyceollin in the roots (Adss et al., 2021). This phytoalexin can contribute to containing the development of the root lesion nematode, and at 56 days with well-established colonization, the fungus may have greater resistance to the antimicrobial effects of glyceolins, allowing this increase in the roots.

At the beginning of *P. brachyurus* infection, at 7 days, *Pochonia chlamydosporia* contributed to the increase of medicarpin in both cultivars. Tests *in vitro* with this compound inhibited *P. penetrans* motility (Baldridge et al., 1998). Thus, roots with a higher concentration of this compound may hinder the movement of *P. brachyurus.*

Phytolalexin coumestrol is related to resistance mechanisms in soybeans in situations of biotic stress. *P. penetrans* infected soybean after treatment with N-3-oxo-tetradecanoyl-Lhomoserin lactone increased coumestrol synthesis in roots (Adss et al., 2021). In lima bean roots at the beginning of *P. scribneri* infection, there was a significant increase in coumestrol, and *in vitro* tests confirmed the effect of this compound in reducing *P. scribneri* motility (Rich et al., 1977). Thus, a possible defense mechanism of soybean when infected by *P. brachyurus* is the increase in the synthesis of coumestrol. In the cultivar BRS 7380 with *P. chlamydosporia*, this compound was reduced in the period of 7 and 21 days, which may have contributed to the highest reproduction of *P. brachyurus* in this treatment. On the other hand, the roots of cultivar MG/BR 46 with the fungus contained a greater amount of coumestrol at 56 days, which may have been essential to contain the development of the nematode, contributing to the reduction of the final population.

5. Conclusion

The use of microorganisms is an efficient and sustainable method for controlling plant parasitic nematodes, as observed for soybean plants inoculated with *P. chlamydosporia*. However, this effect was genotype-dependent because the presence of the fungus reduced the nematode number in the roots of the MG/BR 46 and increased for BRS 7380 genotype. The phytohormonal profiles were also distinct between genotypes, indicating different response cascades of the plants under fungal colonization and nematode attack. The comparison of the response of the two cultivars infected by *P. brachyurus* in the presence of *P. chlamydosporia* allowed us to conclude that in MG/BR 46, the reductions in the concentration of hormones, flavonoids, phytoalexins, and phenolics may be necessary for better colonization of the fungus, mainly the that occurred at 7 days. Once the microorganism is well established, the induction of other defense mechanisms will occur since, in this cultivar, the fungus was efficient in controlling it. Thus, this study of the interaction between soybean, *P. brachyurus*, and *P. chlamydosporia* showed the complexity of the interactions for a microorganism to help control the pathogen and that the reductions in different root compounds do not indicate greater susceptibility to the pathogen, even in compounds with the potential of the nematicidal activity.

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